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MODULATION OF THE VASCULAR FUNCTION BY CANNABINOIDS, CALCITONIN GENE RELATED PEPTIDE AND CONNEXIN HEMICHANNELS

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Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
$[Ca^{2+}]_e$	Extracellular calcium concentration
Apaf	Apoptosis activating factor
ATP	Adenosine 5'-triphosphate
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
Bid	BH3 interacting-domain death agonist
BK_{Ca}	Large conductance calcium-activated potassium channel
cADPR	Cyclic adenosine diphosphate ribose
cAMP	Cyclic adenosine monophoshate
CB1	Cannabinoid 1 receptor
CB2	Cannabinoid 2 receptor
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene related peptide
CICR	Calcium-induced calcium release
COX	cyclooxygenase
CPA	Cryoprotecting agent
c-Src	Cellular sarcoma
Cx	Connexin
CxHC	Connexin hemichannel
CytC	Cytochrome C
DAG	diacylglycerol
dATP	Deoxy adenosine 5'-ATP
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
EC	Endothelial cell
EDHF	Endothelial derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase

ER	Endoplasmic reticulum
ERAD	ER-associated degradation pathway
ERK	Extracellular signal-regulated kinase
GJ	Gap junction
GJIC	Gap junctional intercellular communication
IICR	IP_3-induced calcium release
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
K_{ATP}	ATP sensitive potassium channel
MAPK	Mitogen-activated protein kinase
MEJ	Myoendothelial gap junction
NAADP	Nicotinic acid adenine dinucleotide
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
Panx	Pannexin
PGI_2	prostacyclin
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase C
PKA	Protein kinase A
PKB	Protein kinase B
PPAR	Peroxisome proliferator activated receptor
PS	Phosphatidylserine
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SERCA	Sarco-endoplasmic reticulum Ca^{2+}-ATPase
sGC	Soluble guanylyl cyclase
SMC	Smooth muscle cell
SR	Sarcoplasmic reticulum
Src	Sarcoma
TAT	Trans-activator of transcription
TEA	Tetraethylammonium

THC	Δ^9-tetrahydrocannabinol
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
TRP	Transient receptor potential ion channels
TRPV1	Type 1 vanilloid receptor
TRPV4	Type 4 vanilloid receptor
UTP	Uridine triphosphate
VDCC	Voltage dependent calcium channels
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
ZO-1	Zonula occludens 1

Summary

Blood vessel diameter and blood flow are tightly controlled by electrical signals and calcium ion (Ca^{2+}) signals in smooth muscle cells (SMCs) and endothelial cells (ECs) in which selective and non-selective ion channels play a crucial role. In this doctoral thesis we characterized the targets of cannabinoid- and calcitonin gene related peptide (CGRP)-triggered vasodilation and the role of connexin channels in the modulation of blood vessel tone. Additionally, we investigated connexin channels as a putative target to protect blood vessels against cell death associated with stressful conditions like cryopreservation.

Blood vessel tonus can be modulated by several factors, such as endothelial factors or factors released from perivascular nerves, thereby promoting vasodilation or vasoconstriction. Cannabinoids have been used for centuries for their psychoactive properties, but they have also a profound influence on the cardiovascular system, leading to vasodilation. Vasorelaxation by cannabinoids can be mediated by stimulation of cannabinoid receptors. Others pointed a role for vanilloid receptors on perivascular nerves and a subsequent release of the neuropeptide CGRP. This peptide is one of the most potent vasodilatory substances and can act *via* an endothelium-dependent or -independent process. In order to find out if a direct effect of the cannabinoid methanandamide was present in small mesenteric rat arteries, we performed experiments on acutely isolated SMCs. Our findings show that the cannabinoid methanandamide fails to increase the membrane potassium (K^+) currents and fails to hyperpolarize the membrane potential. Moreover, the neuropeptide CGRP is acting specifically and directly on these cells by increasing the large-conductance calcium-dependent potassium (BK_{Ca}) channel activity in a receptor-, cyclic adenosine monophosphate- (cAMP), and protein kinase A- (PKA) dependent way and hyperpolarizes the membrane potential of these cells. This is consistent with earlier reports suggesting that methanandamide relaxes and hyperpolarizes intact rat mesenteric arteries by releasing the neuropeptide CGRP from perivascular nerves.

Next to electrical changes, vessel diameter and blood flow are also controlled by Ca^{2+} signals and communicative pathways between vascular cells in which gap junctions (GJs) play a crucial role. GJs are dodecameric channels composed of connexin proteins that directly connect the cytoplasm of cells. These intercellular channels are composed of two hexameric

hemichannels (HCs) that may also be junctional as non-junctional HCs. Non-junctional (unapposed) HCs are present in the plasma membrane normally closed but may open by various messengers and conditions thereby forming a pore that allows passage of ions and messengers like ATP or other substances with a molecular weight below 1.5 kDa. HC opening allows Ca^{2+} entry into the cells but opening is also controlled by the intracellular (cytoplasmic) Ca^{2+} concentration ($[Ca^{2+}]_i$). Therefore, these channels are likely to contribute to Ca^{2+} oscillations which are repetitive $[Ca^{2+}]_i$ changes. We investigated the role of HCs in Ca^{2+} oscillations by interfering with HC function *via* the application of peptides that are identical to connexin sequences on the connexin protein. We found that in small mesenteric artery fragments isolated from rat, norepinephrine-induced Ca^{2+} oscillations in SMCs were inhibited by the connexin mimetic peptide Gap27, by the TAT-L2 peptide and by the TAT-CT9 peptide. Gap27 inhibits HCs first and with some delay also GJs; it targets multiple connexins because it mimics well-conserved extracellular domains of the connexin protein. By contrast, TAT-L2 peptide has specificity at two levels: it only inhibits HCs but not GJs and it has specificity for Cx43 and not for other connexins. The latter is related to the fact that this peptide is identical to a part of the intracellularly located non-conserved domain. TAT-CT9 also has specificity for Cx43 but this peptide prevents the closure of HCs at high micromolar $[Ca^{2+}]_i$. In addition to allowing Ca^{2+} entry, open HCs can also allow the escape of ATP from the cell. We found that interfering with purinergic signaling, by promoting the degradation of ATP or by inhibiting purinergic receptors, norepinephrine-induced Ca^{2+} oscillations were also inhibited. Taken together, these findings suggest that ATP released by SMCs *via* connexin hemichannels (CxHCs) with subsequent activation of purinergic communication is involved in controlling vessel responses to norepinephrine. Tension measurements further confirmed the role of CxHCs in vascular smooth muscle tension since the contractility was also inhibited by Gap27, by TAT-L2 and by purinergic receptor antagonists. These data indicate that interfering with CxHCs offers a novel approach towards modulating blood vessel reactivity.

GJs and CxHCs are important for vascular function, but they also play a role in cell death processes. GJs allow the passage of cell death messengers thereby contributing to the propagation of cell death known as bystander cell death. Unapposed CxHCs may promote cell death by uncontrolled opening and thereby contribute to the entrance or escape from

the cell of ions and small metabolites. Inhibiting connexin channels may thus lead to improved cell viability after exposure to stressful conditions like exposure to freezing conditions in the context of cryopreservation of cells and tissues. Cryopreserved blood vessels are being increasingly employed as grafts in vascular reconstruction procedures. However, the massive cell death provoked by the methods of cryopreservation used at most vascular banks is one of the main factors leading to the failure of grafting procedures performed using cryopreserved vascular allografts. We investigated whether blocking connexin channels could prevent cell death after cryopreservation. We report that Gap27 significantly reduced cell death in human femoral arteries and saphenous veins when present during cryopreservation/thawing. Veins had a better conserved intimal layer compared to arteries and had less endothelial cell death. This implies that veins are better suited for vessel replacement in patients with vascular diseases and a high risk of thrombosis because of the anti-thrombogenic effect of an intact endothelium. Most notably, blocking GJs and HCs with connexin mimetic peptides like Gap27 appears to be very efficient to prevent cell death in complex tissues like blood vessels undergoing cryopreservation. It is important to note here that blocking GJs and HCs is (in most cases) are not indicated *in vivo* because GJs are necessary for normal physiological function. However, inhibition of GJs and HCs is perfectly possible in tissues and organs isolated ex vivo for subsequent cold or cryo storage. Such procedure may well be indicated in the context of heart, lung and kidney transplantation and cryopreservation/vitrification of various cells and tissues.

In conclusion, this work has improved our understanding of the vascular action of cannabinoids and CGRP, and has brought up connexins and their channels as interesting new targets to modulate blood vessel function and to protect vascular cell viability after cryopreservation.

Samenvatting

De diameter van een bloedvat en de bloeddoorstroming zijn strikt gereguleerd door elekrische signalen en calcium ion (Ca^{2+}) signalen in gladde spiercellen en endotheelcellen waarin selectieve en niet-selectieve ionkanalen een cruciale rol spelen. In deze doctoraatsthesis karakteriseren we de doelwitten van cannabinoïde- en calcitonine gene related peptide (CGRP)-getriggerde vasodilatatie en de rol van connexine kanalen in de modulatie van de contractiegraad van een bloedvat. Bijkomend onderzochten we Cx kanalen als een mogelijks doelwit om bloedvaten te beschermen tegen celdood die geassocieerd wordt met stressvolle omstandigheden zoals cryopreservatie.

De contractiegraad van een bloedvat kan gewijzigd worden door verschillende factoren die aanwezig zijn in het endotheel of factoren die vrijgesteld worden uit zenuwen gelegen rond een bloedvat (perivasculaire zenuwen). Cannabinoïden worden al eeuwen gebruikt omwille van hun psychoactieve eigenschappen, maar ze hebben ook een grote invloed op het cardiovasculair systeem en leiden tot een vasodilatatie. Deze vasodilatatie door cannabinoïden kan gemedieerd worden door een stimulatie van cannabinoïd receptoren. Daarnaast kunnen ook vanilloïde receptoren die gelegen zijn op de perivasculaire zenuwen een vasodilatie bewerkstelligen en *via* deze weg vasodilaterende substanties vrijstellen zoals het neuropeptide CGRP. Dit peptide staat bekend als één van de meest potente vasodilatoren en kan inwerken *via* het endotheel of rechtstreeks op de gladde spiercel. Om een direct effect van het cannabinoïd methanandamide na te gaan, gebruiken we acuut geïsoleerde kleine mesenterische gladde spiercellen van de rat. We vonden geen rechtstreeks effect van het cannabinoïd methanandamide op de kalium (K^+) stromen of de membraanpotentiaal; wat doet vermoeden dat het effect van methanandamide onrechtstreeks gebeurt, *via* de vrijstelling van CGRP op perivasculaire zenuwen. Inderdaad, CGRP werkt rechtstreeks op de gladde spiercellen in en dit vertaalt zich door een stijging van de K^+ stromen en een hyperpolarizatie van de membraanpotentiaal. De calciumafhankelijke K^+ kanalen (BK_{Ca}) worden geactiveerd *via* een receptor-, cAMP- en PKA-afhankelijke weg. We kunnen dus besluiten dat methanandamide kleine mesenterische bloedvaten relaxeert door CGRP vrij te stellen uit de perivasculaire zenuwen.

Naast elektrische veranderingen, worden de bloeddoorstroming en de bloedvat diameter ook gereguleerd door Ca^{2+} signalen en signaaltransductiewegen tussen vasculaire cellen waarin gap juncties een belangrijke rol spelen. Gap juncties zijn kanalen die bestaan uit 12 connexine eiwitten die een directe verbinding maken met het cytoplasma van cellen. Deze intercellulaire kanalen zijn gevormd uit twee hexamere hemikanalen. Niet-geconnecteerde hemikanalen zijn aanwezig in de celmembraan in een gesloten status maar kunnen openen door verschillende stimuli en condities en kunnen de passage toelaten van ionen en boodschapperstoffen zoals ATP en andere moleculen met een moleculair gewicht kleiner dan 1.5 kDa. Door het openen van hemikanalen kan Ca^{2+} de cel binnenkomen maar het openen is ook gereguleerd door de intracellulaire calciumconcentratie ($[Ca^{2+}]_i$). Deze kanalen zijn daardoor ook mogelijks betrokken bij Ca^{2+} oscillaties; dit zijn repetitieve $[Ca^{2+}]_i$ veranderingen. We onderzochten de rol van hemikanalen in Ca^{2+} oscillaties door te interfereren met de hemikanaal functies *via* de toediening van peptiden die identiek zijn aan connexine sequenties op het connexine eiwit. We vonden dat in gladde spiercellen van kleine mesenterische rat arteriën norepinefrine-geïnduceerde Ca^{2+} oscillaties geïnhibeerd werden door het connexine mimetische peptide Gap27, door het TAT-L2 peptide en door het TAT-CT9 peptide. Gap27 inhibeert eerst hemikanalen en in een latere fase gap juncties; het is een doelwit voor verschillende connexines omdat het een goed bewaard extracellulair domein van het connexine eiwit nabootst. Het TAT-L2 peptide daarentegen, heeft een specificiteit op twee niveaus: het inhibeert enkel hemikanalen en het heeft een specificiteit voor Cx43 en niet voor andere connexines. Dit komt omdat het peptide identiek is aan een deel van het niet-geconserveerd intracellulair domein. TAT-CT9 is ook specifiek voor Cx43 maar dit peptide verhindert het sluiten van de hemikanalen bij een hoge micromolaire $[Ca^{2+}]_i$. Naast het binnenkomen van Ca^{2+}, kunnen hemikanalen ook de loslating van ATP naar buiten toelaten. We vonden dat door het interfereren met purinerge signaaltransductiewegen *via* een degradatie van ATP of *via* het inhiberen van purinerge receptoren, ook de norepinefrine-geïnduceerde Ca^{2+} oscillaties geïnhibeerd werden. Tot slot, suggereren deze bevindingen dat ATP dat vrijgesteld wordt uit gladde spiercellen *via* connexine hemikanalen en de activatie van het purinerge systeem een rol speelt in de controle van de norepinefrine-getriggerde bloedvatrespons. Tensiemetingen bevestigden verder de rol van hemikanalen in vasculaire gladde spier tonus gezien de contractiliteit geïnhibeerd werd door Gap27, TAT-L2 en purinerge receptor antagonisten. Deze data tonen

aan dat het interfereren met connexine hemikanalen een nieuwe mogelijkheid biedt om de reactiviteit van een bloedvat te wijzigen.

Gap juncties en connexine hemikanalen zijn niet alleen belangrijk voor de vasculaire functie, maar dragen ook bij tot celdoodprocessen. Gap juncties laten de passage van celdood boodschappers door, wat bijdraagt tot de verspreiding van celdood. Connexine hemikanalen die niet als gap juncties zijn geconnecteerd, zullen bij ongecontroleerd opengaan celdood promoten en zo ionen en kleine metabolieten zowel in als uit de cel doorlaten. Het blokkeren van connexine kanalen kan dus leiden tot een verbeterde celviabiliteit na blootstelling aan stresvolle omstandigheden zoals het invriezen van cellen en weefsels. Ingevroren bloedvaten worden gebruikt om een aangetast bloedvat te vervangen bij een patiënt. Echter, de methodes om bloedvaten in te vriezen en te ontdooien zijn niet optimaal gezien de grote celdood in de bloedvaten na preservatie, en dit leidt tot het falen van de vasculaire greffe. We onderzochten het effect van Gap27, dat tijdens het invriezen en ontdooien werd toegevoegd aan het preservatiemedium en vonden een significante reductie in celdood in zowel humane dijbeenslagaders (arteria femoralis) als stamaders (vena saphena). Aderen of venen hadden een beter bewaard endotheel en minder celdood in die laag in vergelijking met slagaderen. Dit betekent dat gezien de antitrombotische eigenschappen van het endotheel, dat bij patiënten met vaatziektes die een groter risico hebben op een trombose, een vene met intact endotheel de voorkeur geniet bij transplantatie. Het blokkeren van gap juncties en hemikanalen met connexine mimetische peptiden zoals Gap27 blijkt heel efficiënt te zijn in de preventie van celdood in complexe weefsels zoals ingevroren bloedvaten. Het is belangrijk te vermelden dat het blokkeren van gap juncties en hemikanalen in de meeste situaties niet aangewezen is *in vivo* omdat gap juncties belangrijk zijn voor een normale fysiologische functie. Maar inhibitie van gap juncties en hemikanalen is perfect mogelijk in geïsoleerde weefsels en organen die koel worden bewaard of ingevroren. Zo een procedure kan aangewezen zijn bij een hart-, long- en levertransplantatie of bij het invriezen van verschillende cellen en weefsels.

Tot slot heeft dit werk onze kennis verruimd over de vasculaire actie van cannabinoïden en CGRP, en heeft het connexines en zijn kanalen naar voor gebracht als mogelijke nieuwe doelwitten om de bloedvat functie te moduleren en de celviabiliteit te beschermen na invriezen.

Preface

Vascular tone plays an important role in the regulation of blood pressure and the distribution of blood flow between and within tissues and organs of the body. Blood flow is dependent on small blood vessel diameter. Regulation of the contractile activity of vascular smooth muscle cells (VSMCs) in the systemic circulation is dependent on a complex interplay of vasodilator and vasoconstrictor stimuli from circulating hormones, neurotransmitters, endothelium-derived factors and blood pressure. Like all muscle cells, vascular smooth muscle uses Ca^{2+} as a trigger for contraction. Ca^{2+} entry through plasma membrane channels and Ca^{2+} release from intracellular stores are the major sources of Ca^{2+} leading to blood vessel contraction (Berridge 2008). In addition, the movement of ions through ion channels determines, to a large extent, the membrane potential. The membrane potential, along with the cytosolic Ca^{2+} concentration, regulates and modulates the influx and release of Ca^{2+} through ion channels and the sensitivity of the contractile machinery to Ca^{2+} (Thorneloe and Nelson 2005). K^+ channels are the predominant ion conductive pathways in VSMCs. As such, their activity importantly contributes to determine and regulate the membrane potential and vascular tone (Ko *et al.* 2008). The electrochemical gradient for K^+ ions is such that opening of K^+ channels results in diffusion of this cation out of the cells thereby hyperpolarizing the membrane. A hyperpolarization of the membrane potential closes voltage-gated Ca^{2+} channels and leads to vasodilation, whereas depolarization opens them, with a vasoconstriction as result. Thus, by their predominant role in setting the membrane potential, K^+ channels play a central role in the regulation of vascular tone. Other channels that may influence the membrane potential and $[Ca^{2+}]_i$ are the connexin channel family, consisting of GJs and HCs. GJ channels connect SMCs among each other and SMCs with ECs. These channels allow the passage of ions and metabolites with a molecular weight below 1.5 kDa. Apart from this, unapposed non-junctional HCs in the plasma membrane connect between the cytoplasma and the extracellular space. Uncontrolled opening of CxHCs can lead to an ionic imbalance and this might contribute to cell death and cell survival mechanisms.

In this doctoral thesis, we investigated the role of cannabinoids and CGRP on SMC K^+ channels and determined the role of CxHCs on $[Ca^{2+}]_i$ dynamics and contraction. While GJs and HCs play a role in vessel physiology SMCs, they may also contribute to cell death under

stressful conditions. We further determined whether inhibition of connexin channels, i.e. GJs and HCs, could protect blood vessels against cryoinjury. In the following 3 chapters we introduce each of these topics. In chapter IV we discuss our aims and in chapter V to VII our results. The general discussion and future perspectives will be discussed in Chapter VIII.

Chapter I: Cannabinoids and calcitonin gene related peptide modulating the vessel wall reactivity

1. Cannabinoids

1.1. Introduction

Plant-derived cannabinoids such as Δ^9-tetrahydrocannabinol (THC), the active ingredient of the hemp plant, have been used for centuries for their psychoactive properties (Hiley and Ford 2004). In the early 1990s, endogenous cannabinoids were described. The first of these endocannabinoids identified was N-arachidonoyl ethanolamide (anandamide) and had pharmacological and behavioural effects similar to THC (Devane *et al.* 1992). Since their discovery, the effects of both synthetic and endogenous cannabinoids have been extensively examined. In addition to their neurobehavioural effects, they have a profound influence on the cardiovascular system (Hillard 2000, Hiley and Ford 2004) and gastrointestinal function (Germano *et al.* 2001). Reported effects include vasodilation in a number of vascular beds, hypotension, bradycardia, inhibition of gastrointestinal motility and of gastric acid secretion and a reduction of stress-induced gastric ulcers (Kunos *et al.* 2000, Germano *et al.* 2001, Pertwee 2001, Hiley 2009).

Endocannabinoids are synthesized in several cell types to regulate the cardiovascular system: in vascular SMCs, endothelial cells, nerve cells within the blood vessel walls (i.e. perivascular nerves) and circulating cells of the blood (Ishioka and Bukoski 1999, Hogestatt and Zygmunt 2002, Hiley and Ford 2004, Mach and Steffens 2008). The endocannabinoid anandamide (Figure 1) has been identified as an endogenous counterpart of THC, the psychoactive component of marijuana. The endocannabinoid is synthesized by cleavage of the lipid precursor arachidonyl phosphatidylethanolamine, and is metabolized relatively quickly to arachidonic acid and ethanolamine. Low levels of anandamide have been detected in rat blood and human serum (Giuffrida and Piomelli 1998, Wang *et al.* 2001). Another interesting source of anandamide in the cardiovascular system is circulating macrophages (monocytes), which can produce anandamide *in vitro* and in pathological conditions such as haemorrhagic and endotoxic shock, and advanced liver cirrhosis (Varga *et al.* 1998, Batkai *et al.* 2001, Wang *et al.* 2001). Substantial levels of anandamide have been detected in various animal tissues including skin and brain (Calignano *et al.* 1998, Bisogno *et al.* 1999). Tissue macrophages within and surrounding the vascular wall may produce anandamide locally and could be an important source of anandamide in atherosclerosis and inflammation.

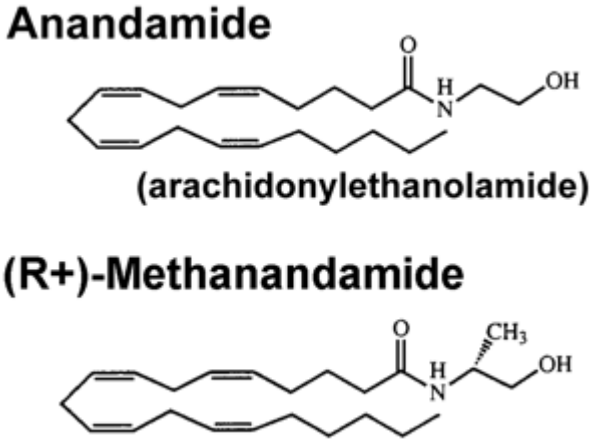

Figure 1. Structures of the endocannabinoid anandamide and its stable analogue methanandamide. (Vanheel and Van de Voorde 2001)

1.2. Receptors

a) Cannabinoid (CB) receptors

The classical receptors for cannabinoids are the type 1 cannabinoid (CB1) and type 2 cannabinoid (CB2) receptors and both receptor subtypes are coupled through G-proteins to adenylyl cyclase and mitogen-activated protein kinase (MAPK). Cannabinoid CB1 receptors are mainly localized on neurons but are also present in some peripheral tissues including heart, lung and gastrointestinal tissues and in vasculature (Howlett *et al.* 2010). In several arteries, the vasorelaxant effect of cannabinoids is mediated by stimulation of CB1 receptors. Indeed, SR141716A, a CB1 receptor antagonist, inhibits relaxations by anandamide in rat mesenteric arteries, coronary arteries and kidney arterioles (Randall *et al.* 1996, Deutsch *et al.* 1997, White *et al.* 2001, O'Sullivan *et al.* 2004) and in cat cerebral arteries (Gebremedhin *et al.* 1999). Additionally, the hypotensive action of anandamide has been shown to be absent in mice lacking CB1 receptors (Ledent *et al.* 1999). Stimulation of the CB1 receptor modulates the activity of several types of ion channels *via* G-proteins. For example, it has been shown that (endo)cannabinoids block voltage-gated N-, L-, T and P/Q-type Ca^{2+} channels (Mackie and Hille 1992, Twitchell *et al.* 1997, Gebremedhin *et al.* 1999) and stimulate inwardly rectifying K^+ channels (Henry and Chavkin 1995, Mackie *et al.* 1995). Moreover, endocannabinoids can directly target and inhibit voltage-gated Ca^{2+} channels (Oz *et al.* 2000, Chemin *et al.* 2001), Na^+ channels (Nicholson *et al.* 2003) and various types of K^+

channels (Poling *et al.* 1996, Van den Bossche and Vanheel 2000, Maingret *et al.* 2001), although usually at higher concentrations.

CB2 receptors are primarily expressed in immune and hematopoietic cells (Munro *et al.* 1993), although there is some evidence that these receptors are also expressed by neuronal tissue (Howlett *et al.* 2010) and recent evidence indicates a relatively widespread yet sparse distribution in the brain (Onaivi *et al.* 2006). There is little evidence of an involvement of CB2 receptors in cannabinoid modulation of perivascular neurotransmission, although CB2 activation has been reported to inhibit murine mesenteric afferent nerve activity (Hillsley *et al.* 2007). Recent evidence suggests that, at least in some vessels, the vasodilator response of cannabinoids is caused by stimulation of an endothelial non-CB1/CB2 receptor (CB_x) (Jarai *et al.* 1999, Ford *et al.* 2002, Herradon *et al.* 2007)(Figure 2).

b) TRPV receptors

The vasorelaxant influence of cannabinoids has also been reported to be due to stimulation of type 1 vanilloid (TRPV1) receptors on primary afferent perivascular nerves and the subsequent release of sensory neuropeptides such as the powerful vasodilator CGRP (Figure 2). In rat hepatic and small mesenteric arteries, the vasorelaxant influence of the cannabinoid anandamide was antagonized by the TRPV1 receptor antagonist capsazepine and the CGRP receptor antagonist CGRP(8-37), but not by the CB1 receptor antagonist SR141716A (Zygmunt *et al.* 2002), pointing to a role for the TRPV1 receptor in vasodilation by anandamide. The vanilloid TRPV1 receptor is a non-selective cation channel that displays a high permeability for Ca^{2+}, and acts as a polymodal nociceptor, being activated by a variety of noxious stimuli including capsaicin, heat (> 42 °C), protons (pH < 6.5) and endogenous cannabinoids such as anandamide and N-arachidonoyl-dopamine (Caterina *et al.* 1997, Szallasi and Blumberg 1999). The receptor was originally identified as the recognition site for capsaicin, the pungent ingredient in hot chilli peppers. Although low levels of expression have been detected in certain brain areas, the vanilloid receptor is expressed predominantly on a population of capsaicin-sensitive primary afferent nerve fibers, namely thin, unmyelinated C-fibres and large diameter myelinated Aδ fibres, which are activated by a variety of noxious stimuli such as thermal, chemical and mechanical stress. These nerve fibres also participate in visceral reflexes and in local responses to tissue injury. Sensory

neurons from TRPV1 gene knockout mice have an impaired response to heat, protons and capsaicin, and initial studies have demonstrated that these animals fail to develop inflammation-induced thermal hyperalgesia (Holzer 1992, Szallasi and Blumberg 1999). Much interest has been focused on the role of the vanilloid receptor in pain signaling and inflammation, in which vasodilation is an important component (Hogestatt and Zygmunt 2002).

The picture is further complicated by more recent studies that identified endocannabinoids as potential activators of the type 4 vanilloid (TRPV4) receptor (Watanabe *et al.* 2003)(Figure 2). The receptor is highly expressed on endothelial cells and its stimulation enhances Ca^{2+} influx, an essential trigger to release various vasoactive factors including nitric oxide (NO), prostaglandins, and to activate the endothelial derived hyperpolarizing factor (EDHF) mechanism (Yao and Garland 2005). Moreover, TRPV4 was also found in myocytes of cerebral arteries, and its activation was shown to cause SMC hyperpolarization *via* BK_{Ca} channel activation (Earley *et al.* 2005).

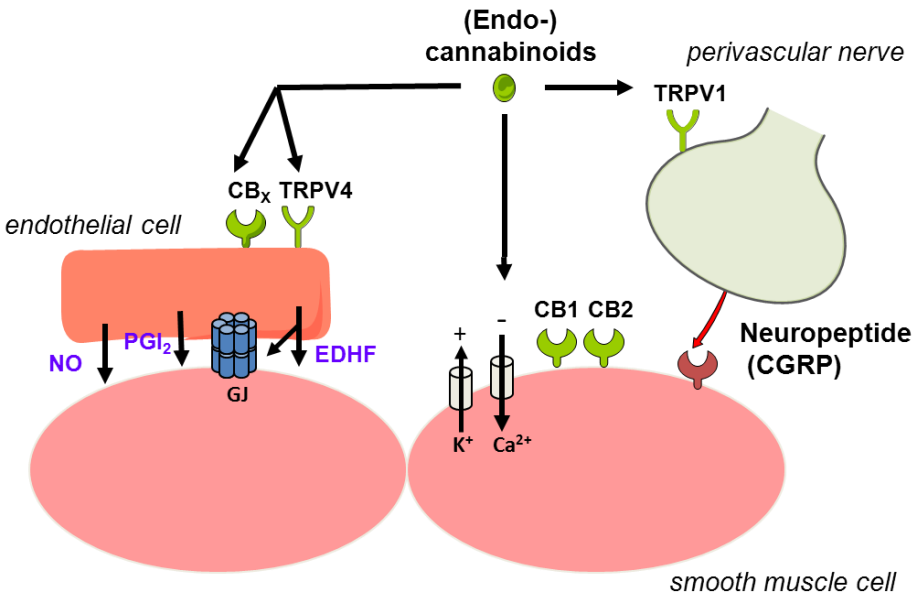

Figure 2. Schematic presentation of the possible mechanisms of action of (endo)cannabinoids in the blood vessel wall. NO = nitric oxide; PGI_2 = prostacyclin; GJ = gap junction; EDHF = endothelial derived hyperpolarizing factor; CGRP = calcitonin gene related peptide; CB1 = type 1 cannabinoid receptor; CB2 = type 2 cannabinoid receptor; CB_x = non CB1/CB2 cannabinoid receptor; TRPV1 = type 1 vanilloid receptor; TRPV4 = type 4 vanilloid receptor.

c) Other receptors

Several cannabinoid receptor ligands also bind to the orphan G-protein-coupled receptor GPR55 suggesting that this might represent an additional receptor subtype (Ryberg *et al.* 2007, Lauckner *et al.* 2008). Activation by THC or methanandamide, a stable analogue of anandamide, increases the $[Ca^{2+}]_i$ in neurons expressing this GPR55 receptor (Lauckner *et al.* 2008). It has become evident that some cannabinoids, in addition to their effects on G protein-coupled receptors and TRP ion channels, can activate members of the nuclear peroxisome proliferator activated receptor (PPAR) family (O'Sullivan *et al.* 2005, Sun and Bennett 2007). THC was shown to be a $PPAR_\gamma$ ligand and $PPAR_\gamma$ agonists are used in the management of type 2 diabetes to improve insulin sensitivity and further stimulate vasorelaxation, increased availability of NO, anti-inflammatory actions, attenuation of atherosclerosis and vascular remodelling (O'Sullivan *et al.* 2005). Furthermore, an action on other receptors was suggested. Anandamide has been demonstrated to bind to serotonin (5-HT)-3 receptors (Fan 1995), thereby contributing to the effects of cannabinoids, such as antiemesis and analgesia, and modulate the activity of the *N*-methyl-D-aspartate (NMDA) receptor (Hampson *et al.* 1998). Other potential receptors for anandamide are the muscarinic and nicotinic acetylcholine receptor, the glycine receptor, Ca^{2+}, K^+ and Na^+ channels (Di Marzo *et al.* 2002).

In table 1 an overview is shown of the receptors and their location for anandamide/methanandamide.

Table 1. Overview of the receptors and their location for anandamide/methanandamide.

Receptor	Location
CB1 (Howlett *et al.* 2010)	Neurons, heart, lung, gastrointestinal tissues, vasculature
CB2 (Munro *et al.* 1993)	Immune and hematopoietic cells, neuronal tissue, brain
Non-CB1/CB2 (CB_x) (Jarai *et al.* 1999, Ford *et al.* 2002, Herradon *et al.* 2007, McCollum *et al.* 2007, Baranowska-Kuczko *et al.* 2012)	Endothelial cells
TRPV1 (Zygmunt *et al.* 1999)	Perivascular nerves, brain, spinal cord, bladder
TRPV4 (Earley *et al.* 2005, Yao and Garland 2005)	Endothelial cells, myocytes of cerebral arteries
TRPM8 (De Petrocellis *et al.* 2007, De Petrocellis *et al.* 2008)	Neurons, prostate, lungs, bladder
TRPA1 (De Petrocellis and Di Marzo 2009)	Neuronal tissue, pancreatic cells, endothelial cells, perivascular nerves
GPR55 (Lauckner *et al.* 2008)	Brain, vascular endothelium, vascular smooth muscle, immune system

PPARα (Bouaboula *et al.* 2005, Sun and Bennett 2007)	Liver, heart, muscle, kidney and monocyte-derived macrophages, smooth muscle cells and endothelial cells of the arterial wall
PPARγ (Sun and Bennett 2007)	Adipose tissues, colonic epithelia, endothelial cells, macrophages
Serotonin (5-HT)-3 receptor (Fan 1995)	Peripheral and central nervous system
NMDA receptor (Hampson *et al.* 1998)	Central nervous system, heart, stomach, pancreas, kidney, lung, thymus and stomach
Muscarinic acetylcholine receptor (Lagalwar *et al.* 1999)	central and peripheral nervous system, heart, vascular smooth muscle, lungs
Nicotinic acetylcholine receptor (Oz *et al.* 2003)	Neurons, endothelial cells, smooth muscle cells, neuromuscular junction
Glycine receptor (Hejazi *et al.* 2006)	Central nervous system, smooth muscle cells, endothelial cells
Calcium channels (VDCC) (Chemin *et al.* 2001)	Muscle cells, glial cells, neurons
Potassium channels (K_{ATP}, K_V) (Poling *et al.* 1996, Oz *et al.* 2007, Barana *et al.* 2010)	All animal cells
Sodium channels (Na_V) (Nicholson *et al.* 2003, Kim *et al.* 2005)	Neurons, myocytes, astrocytes, endothelial cells

1.3. Vasodilatory actions of (meth)anandamide

The potent vasodilatory influence of anandamide has been shown in a variety of isolated vascular preparations. Its mechanism of action, however, is complex and seems to vary with species, vessel type, and even vessel size (Vanheel and Van de Voorde 2001, O'Sullivan *et al.* 2004). Moreover, also methanandamide, is a powerful vasodilator of these vessels (Ralevic *et al.* 2000). In rabbit pial arterioles, rat aorta, rat pulmonary, bovine coronary and rabbit mesenteric arteries, (meth)anandamide produces endothelium-dependent vasodilation *via* cyclooxygenase. In rat mesenteric arteries, however, the vasorelaxing influence of anandamide is unaffected by cyclooxygenase inhibition (Tamaki *et al.* 2012). Additionally, anandamide-induced vasodilation has been reported to be mediated by the release of NO from perfused renal arterial segments (Deutsch *et al.* 1997). Furthermore, others hypothesized that anandamide is produced by ECs and acts as an endothelium-derived hyperpolarizing factor (Randall *et al.* 1997, Randall and Kendall 1998).

Most studies, however, have shown that the vasorelaxations caused by (meth)anandamide are completely (White and Hiley 1998, Breyne *et al.* 2006) or partly (Chaytor *et al.* 1999, O'Sullivan *et al.* 2004) endothelium-independent. This endothelium-independency rules out the possible role of endothelium-derived relaxing factors that have been suggested to be

involved in the cannabinoid-induced relaxations in some isolated vascular preparations (Ellis *et al.* 1995, O'Sullivan *et al.* 2004). The endothelium-independent pathway by which cannabinoids induce vasodilation is still elusive. Some studies report that cannabinoids directly stimulate cannabinoid receptors on the VSMCs (White and Hiley 1998). More recent studies propose that in some vessels, the vasodilator response to cannabinoids is mediated by stimulation of a novel, yet non-identified, non-CB1/CB2 receptor located on the endothelium (Jarai *et al.* 1999, Ford *et al.* 2002, O'Sullivan *et al.* 2004, McCollum *et al.* 2007, Baranowska-Kuczko *et al.* 2012).

Since the original observation that vasorelaxation by cannabinoids can be mediated by stimulation of TRPV1 receptors on CGRP-containing perivascular nerves (Zygmunt *et al.* 1999, Ahluwalia *et al.* 2003, Breyne and Vanheel 2006), several studies have documented TRPV1 stimulation by anandamide or by methanandamide and stressed that anandamide should be considered both an endocannabinoid and an endovanilloid (Ralevic *et al.* 2002, Ahluwalia *et al.* 2003). Previously, Breyne *et al.* found that in gastric arteries a vasorelaxation produced by low concentrations (<1 µM) of methanandamide was completely inhibited after depletion of the perivascular nerves by pre-exposure to capsaicin, a TRPV1 agonist. Moreover, relaxations tended to decrease in the presence of the TRPV1 antagonist capsazepine. These findings suggest that at least at the lower concentrations methanandamide act on capsaicin-sensitive TRPV1 receptors on perivascular nerves and stimulate the release of CGRP. Conversely, relaxations to high concentrations of methanandamide (10 µM) were completely unaffected by 30 mM K^+ and tetraethylammonium (TEA) or glibenclamide, pointing to a mechanism that does not require SMC hyperpolarization, but methanandamide might induce relaxation by reducing calcium entry into the SMCs (Breyne *et al.* 2006). Recently Tamaki *et al.* demonstrated in the rat superior mesenteric artery that anandamide caused a concentration-dependent vascular response by measuring the perfusion pressure: at low concentrations (0.1-1 nM) a vasodilation appeared, while at higher concentrations (10 nM-1 µM) anandamide elicited an initial transient and sharp endothelium-dependent vasoconstriction, which is mediated by CB1 receptors on the vascular endothelium and vasoconstrictor prostanoids, followed by a long-lasting vasodilation mainly mediated by perivascular capsaicin-sensitive CGRPergic nerves (Tamaki *et al.* 2012).

2. Calcitonin Gene Related Peptide

2.1. Introduction

In rat hepatic and small mesenteric arteries, the vasorelaxant influence of anandamide was antagonized by the TRPV1 receptor antagonist capsazepine and the CGRP receptor antagonist CGRP(8-37) but not by the classical CB1 receptor antagonist SR141716A (Zygmunt *et al.* 1999). It was proposed, therefore, that cannabinoids exert their relaxing influence by stimulating the release of CGRP from perivascular nerves (Zygmunt *et al.* 1999, Ahluwalia *et al.* 2003). Figure 3 shows the presence of CGRP in perivascular nerves.

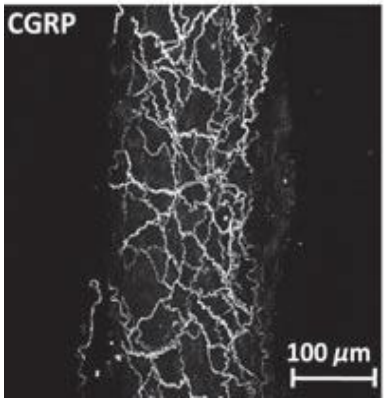

Figure 3. Perivascular nerves containing CGRP. Confocal laser photomicrographic image showing calcitonin gene related peptide (CGRP) in 2nd and 3rd order branches of rat mesenteric arteries. Most blood vessels are surrounded by a dense perivascular CGRP-ergic neural network, which is found at the junction of the adventitia and the media, passing into the muscle layer. (Kawasaki *et al.* 2011)

CGRP is a 37 amino acid peptide that is generated by alternative splicing of the calcitonin gene transcripts. Studies using CGRP-knockout mice have shown that these animals are hypertensive and endowed with an overactivity of the sympathetic nervous system (Oh-hashi *et al.* 2001, Kurihara *et al.* 2003). CGRP is a microvascular vasodilator and is known to play an important role in neurogenic vasodilation in the skin and to increase blood flow in response to injury and noxious stimuli in a variety of pathological and physiological processes including cardioprotection, gastric ulcer protection (Evangelista 2009), migraine, neurogenic inflammation and pain perception (Smillie and Brain 2011). CGRP is widely distributed in the nervous and cardiovascular system and often coexists with substance P. In the peripheral nervous system, the prominent site of CGRP synthesis is the dorsal root ganglion (DRG), which contains the cell bodies of capsaicin-sensitive sensory neurons (Figure 4). The peripheral processes of the sensory neurons terminate on blood vessels and transport CGRP to the nerve endings. CGRP is released from these perivascular nerve

endings in order to promote vasodilation. Their central processes terminate in laminae I and II of the dorsal horn and also synapse with the intermediolateral nucleus of the spinal cord, which contains the sympathetic pre-ganglionic neurons. This connection could influence the activity of the sympathetic nervous system, and thus vascular tone and blood pressure (Deng and Li 2005). Receptors for CGRP have been identified both in the media and intima of resistance vessels (Bell and McDermott 1996).

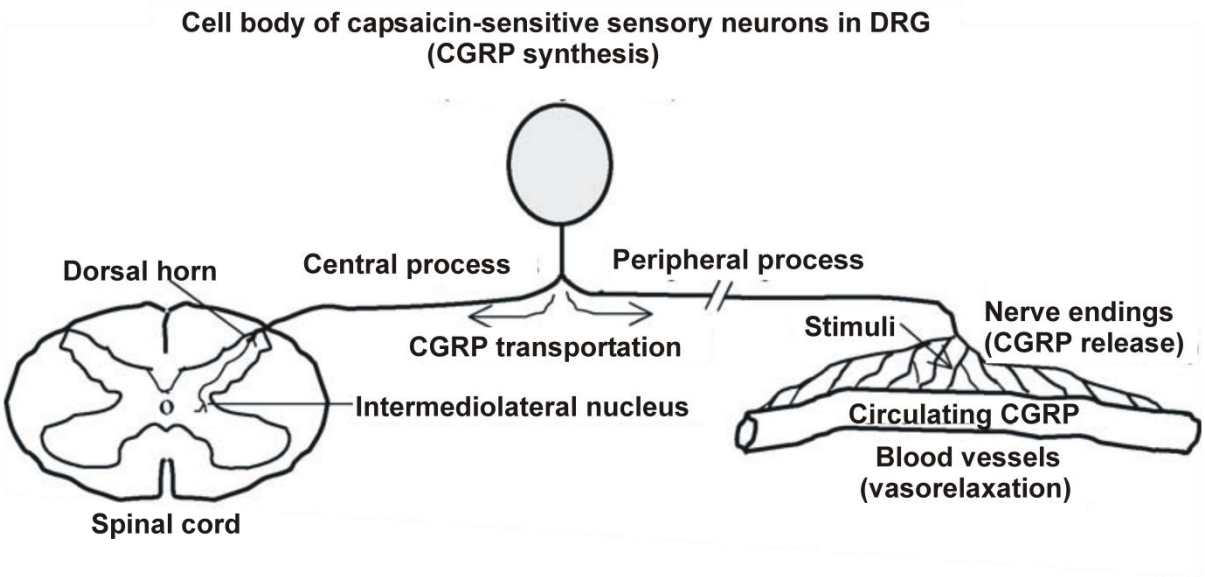

Figure 4. Schematic illustration of the functional anatomy of the capsacin-sensitive sensory neurons, indicating the pathway of CGRP synthesis, release and action. (Figure adapted from Deng *et al.* 2005)

2.2. CGRP receptors

CGRP receptors have originally been classified into two subtypes termed CGRP1 and CGRP2, based on their affinity for CGRP(8-37), a truncated peptide of CGRP. CGRP(8-37) shows a high affinity for the CGRP1 receptor but not for the CGRP2 receptor. The CGRP1 receptor is now considered to be the primary and predominant cardiovascular receptor (Brain and Cox 2006). It is now also apparent that the CGRP2 receptor phenotype is the result of CGRP acting at receptors for amylin and adrenomedulin. Accordingly, the term ”CGRP2” receptor should no longer be used, and the “CGRP1” receptor should be converted to “CGRP” receptor (Hay *et al.* 2008, Walker *et al.* 2010). The CGRP receptor belongs to the family of G-protein coupled receptors. In 1998 McLatchie et al. demonstrated that the calcitonin receptor required co-binding of receptor activity modifying proteins (RAMP) with the

calcitonin like receptor (CLR) in order to form a functional receptor on the cell surface (Figure 5) (McLatchie *et al.* 1998, Conner *et al.* 2007). For functionality an additional protein called receptor component protein (RCP) is required which is essential for effective $G\alpha_s$ coupling (Walker *et al.* 2010). RAMPs are single transmembrane proteins, forming three distinct types, RAMP1, RAMP2 and RAMP3. Of these, CLR linked with RAMP1 leads to a high affinity CGRP receptor (Hay *et al.* 2002). The association of CLR with RAMP2 produces an adrenomedullin 1 receptor, with RAMP3 an adrenomedullin 2 receptor (Brain and Grant 2004, Walker *et al.* 2010) (Figure 5).

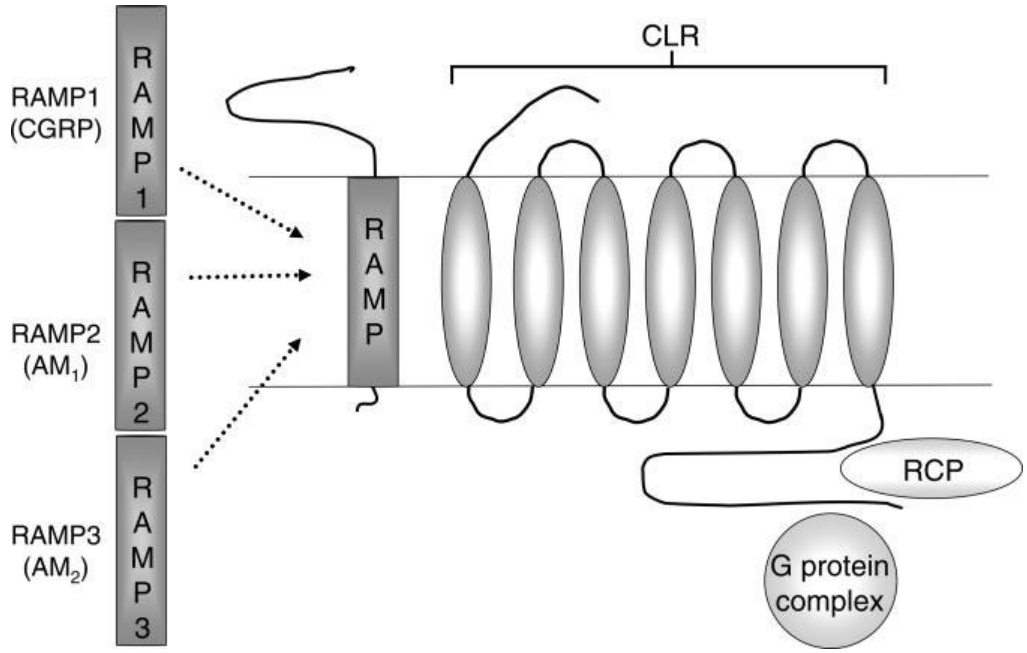

Figure 5. The CGRP/adrenomedullin (AM) receptor. The calcitonin receptor-like receptor (CL) component is common to all three receptors and is a G protein-coupled 7-transmembrane receptor. The three RAMP components are single transmembrane domain proteins. The active receptor is a functional heterodimer of one CL complexed with a RAMP, at the cell membrane. The interaction of RAMP1 with CL produces a CGRP receptor, RAMP2 with CL an AM receptor, and RAMP3 with CL a CGRP/AM receptor. The proposed receptor component protein (RCP), which is suggested to allow coupling to intracellular signaling pathways, is also included. (Brain et al. 2004)

2.3. Vasodilatory actions of CGRP

The mechanism involved in vasorelaxation to CGRP seems to vary among species and vessel types. CGRP mediates endothelium-dependent and -independent relaxation. Vasodilation through an endothelium-dependent pathway is possible through activation of endothelial nitric oxide synthase (eNOS) (release of NO) and soluble guanylate cyclase (sGC) to increase

cyclic guanosine monophosphate (cGMP) (Yoshimoto *et al.* 1998, Hosaka *et al.* 2006)(Figure 6). CGRP–induced vasodilation through an endothelium-dependent pathway involving NO release has been described in rat aorta, mouse pial arteries, rat cremaster venules, rat mesenteric arteries, human mammary arteries and human forearm arteries (Yoshimoto *et al.* 1998, de Hoon *et al.* 2003, Hosaka *et al.* 2006). CGRP-mediated release of NO is very different from that mediated by acetylcholine. Acetylcholine induces synthesis of NO by an increase of endothelial synthesis of IP_3 and intracellular Ca^{2+}. CGRP enhances endothelial production of PKA or protein kinase B (PKB) resulting in an increase in synthesis of NO independent of the increases in intracellular calcium. In rat aorta (Gray and Marshall 1992) and pulmonary arteries (Wisskirchen *et al.* 1998), human omental and bovine retinal arteries (Boussery *et al.* 2005), CGRP interacts with endothelial receptors stimulating the release of endothelium-derived relaxing factors, as both endothelium removal and blocking NO synthesis significantly reduced vasorelaxation.

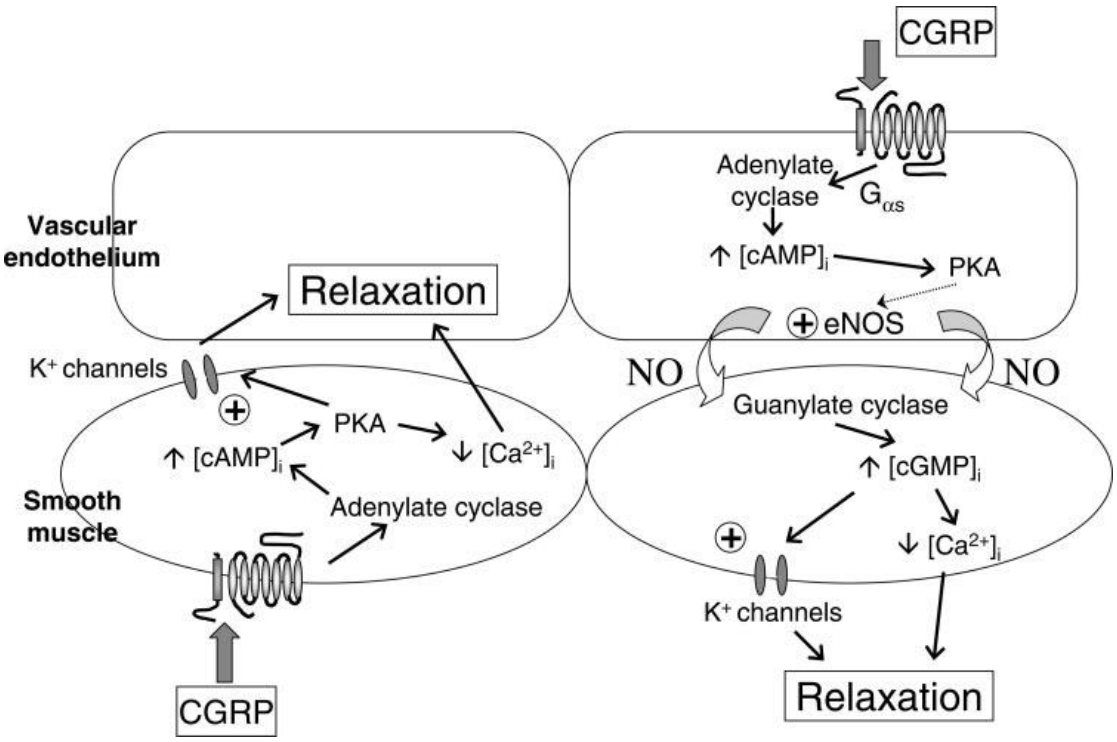

Figure 6. The cellular mechanisms of vasodilation to CGRP. *Left*: endothelium-independent vasodilation to CGRP. Activation of CGRP receptors on smooth muscle cells is coupled to production of cAMP by adenylate cyclase. The increase in intracellular cAMP concentration ($[cAMP]_i$) then stimulates protein kinase A (PKA), which opens K^+ channels and activates Ca^{2+} sequestration mechanisms to cause smooth muscle relaxation. *Right*: endothelium-dependent vasodilation to CGRP. CGRP interacts with receptors on endothelial cells and stimulates production of nitric oxide (NO). This is mediated *via* cAMP accumulation, although a direct effect of PKA on endothelial NO synthase (eNOS) is yet to be fully characterized. Diffusion of NO into adjacent smooth muscle cells, activating guanylate cyclase, then leads to relaxation. (Brain *et al.* 2004.)

Vasodilation through an endothelium-independent process happens through activation of adenylate cyclase to increase cAMP (Figure 6) in the majority of the vessels (rat mesenteric, gastric, splenic and renal arteries, rabbit jejunal, rat and rabbit hepatic artery, porcine coronary arteries, skin and skeletal muscle microcirculations, cat cerebral, dog lingual and human uterine arteries) (Foulkes *et al.* 1991, Nilsson *et al.* 1992, Ishikawa *et al.* 1993, Kageyama *et al.* 1993, Hill and Gould 1997, Yoshimoto *et al.* 1998, Hosaka *et al.* 2006). The resultant activation of PKA and PKA-dependent phosphorylation has been shown to open ATP-sensitive K^+ (K_{ATP}) channels (Table 2) (Nelson *et al.* 1990, Quayle *et al.* 1994, Wellman *et al.* 1998) hyperpolarizing and relaxing the tissue (Wellman *et al.* 1998). Exogenous CGRP produced a hyperpolarization of the SMCs of comparable time course and magnitude to those obtained with methanandamide, and both methanandamide- and CGRP-induced membrane potential changes were sensitive to the K_{ATP} channel inhibitor glibenclamide (Dunn *et al.* 2003, Breyne and Vanheel 2006). However, studies on several vascular beds (Table 2) have not been able to demonstrate a possible involvement of K_{ATP} channel opening in CGRP induced vasorelaxation (Prieto *et al.* 1991, Kageyama *et al.* 1993). CGRP might also mediate relaxation by activation of BK_{Ca} channels (Table 2), by inhibition of voltage gated Ca^{2+} channels (Breyne *et al.* 2006), and by decreasing the Ca^{2+} sensitivity of the contractile apparatus (Sheykhzade and Nyborg 2001). In rat gastric arteries, Breyne *et al.* have found that the vasorelaxation to CGRP was partly sensitive to TEA, suggesting the involvement of BK_{Ca} channel activation (Breyne *et al.* 2006). In a number of vascular preparations, PKA-dependent vasodilators have been shown to activate smooth muscle BK_{Ca} channels (Schubert and Nelson 2001).

Table 2. K^+ channel involvement of CGRP-mediated vasodilatory actions in different vascular beds.

Author	tissue	technique	K^+ channel involved
Nelson M *et al.*, 1990	Rabbit mesenteric arteries	Isometric tension measurements	K_{ATP}
Miyoshi H and Nakaya Y, 1995	Porcine coronary artery	Electrophysiology	K_{Ca}, K_{ATP}
Quayle J *et al.*, 1994	Rabbit mesenteric arteries	Electrophysiology	K_{ATP}
Hill CE and Gould DJ, 1997	Rat irideal arterioles	Electrophysiology and arteriolar diameter measurements	K_{ATP}
Hong K *et al.*, 1996	Rat pial arterioles	Arterial diameter measurements	BK_{Ca}, K_{ATP}
Wellman GC *et al.*, 1998	Porcine coronary artery	Electrophysiology	K_{ATP}
Herzog M *et al.*, 2002	Gerbil spiral modiolar artery	Vascular diameter and $[Ca^{2+}]_i$ measurements	BK_{Ca}, K_{ATP}
Sheykhzade M *et al.*, 2001	Rat coronary artery (precontracted) Rat coronary artery (resting)	Isometric tension and $[Ca^{2+}]_i$ measurements	BK_{Ca}, K_{ATP}
Dunn W *et al.*, 2003	Rat mesenteric arteries	Electrophysiology	K_{ATP}
Dong Y *et al.*, 2004	Umbilical artery/vein, chorionic artery/vein, stem villous vessels	Isometric tension measurements	K_{ATP}
Breyne J *et al.*, 2006	Rat gastric arteries	Isometric tension measurements	BK_{Ca}
Gozalov A *et al.*, 2007	Rat cerebral artery	Isometric tension and arterial diameter measurements	BK_{Ca}
Breyne J and Vanheel B, 2006	Rat gastric and mesenteric arteries	Electrophysiology	K_{ATP}

Chapter II: Connexins, blood vessel function and cell death

1. Connexins: overview

1.1. The connexin family

Connexins are four-pass transmembrane proteins, with two extracellular loops stabilized by intramolecular disulfide bridges, an intracellular loop, and N- and C-terminal cytoplasmic regions (Saez *et al.* 2003, Evans *et al.* 2006). The N-terminus, the two extracellular loops and the four transmembrane domains are highly conserved among different isoforms. In contrast, the cytoplasmic loop and the C-terminus domain are divergent and variable in length and sequence, allowing for the functional differences among the different connexins (Dbouk *et al.* 2009). After two decades of cloning and characterization of the genes encoding connexins, the connexin roster is now likely to be complete with over 20 genes in humans and rodents (Sohl and Willecke 2003). At the onset of connexin biology the nomenclature of connexin proteins was based on the predicted molecular weight of each family member (e.g. Cx43 is ~43kD in size) and this nomenclature continues to be widely used. Another nomenclature proposed a division into subclasses α, β, γ and δ and was based on sequence similarity and length of cytoplasmic domains, using the prefix GJ for gap junction, and a number referring to the order of their discovery. Cx43 is called GJA1 (human) or Gja1 (mouse) (Sohl and Willecke 2003). It is rare for any cell type to express only one member of the connexin family and the coexpression of two or more connexins is the standard. Connexons are formed by six connexin proteins, which assemble into a channel that is often called a hemichannel (HC), with either identical connexin subunits (homomeric) or different connexins (heteromeric) (Figure 1). When two hemichannels from neighboring cells dock, they form GJ channels (Goodenough and Paul 2003). GJ channels may be homotypic, when two identical connexons (or hemichannels) assemble, or heterotypic, when two dissimilar connexons assemble between the two interacting cells (Dbouk *et al.* 2009).

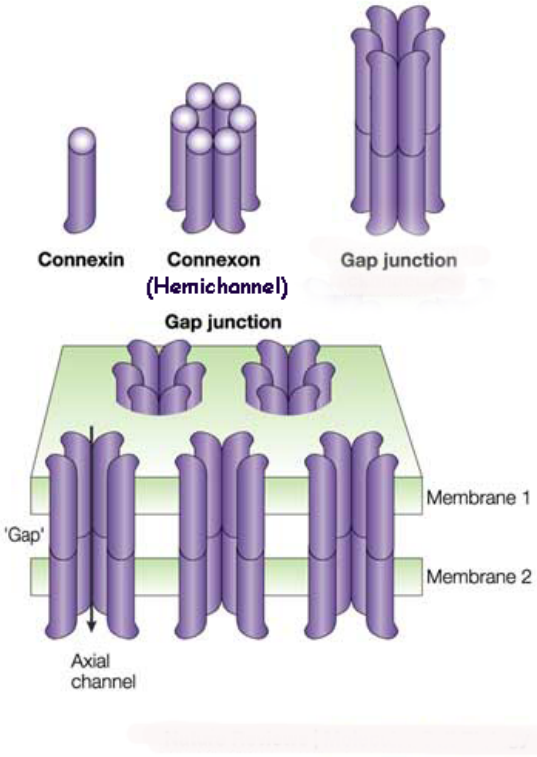

Figure 1. The connexin family. Connexin proteins oligomerize into hexameric connexons or hemichannels and the intercellular joining of two connexons form a dodecameric intercellular channel (gap junction channel). (Figure adapted from Goodenough *et al.* 2003)

1.2. The life cycle of connexins

Connexins are co-translationally inserted into the endoplasmic reticulum (ER) and then trafficked through the Golgi apparatus (Figure 2) (Laird 2006). Misfolded connexins will be directed to the ER-associated degradation pathway (ERAD). At some point during the secretory pathway, connexins oligomerize into hexameric connexons. Depending on the connexin isoform, oligomerization has been observed in various locations ranging from the ER, the ER-Golgi intermediate compartment, to the trans-Golgi network (Diez *et al.* 1999, Koval 2006, Das *et al.* 2009). These connexons, that comprise one-half of the complete GJ channel, are transported to the plasma membrane to form GJs and large accretions of GJs, known as GJ plaques. Recently, undocked connexons at the plasma membrane have been demonstrated to have channel activity, i.e. display channel opening events, as well.

From the plasma membrane, connexins are internalized as double membrane annular GJs, or connexosomes, or possibly, as undocked individual connexons and then degraded (Laird 2006). It is clear that connexins undergo degradation through both the lysosomal and

proteasomal degradation pathways (Laing *et al.* 1998, Leithe and Rivedal 2004, VanSlyke and Musil 2005) and an additional pathway of autophagy was recently revealed (Hesketh *et al.* 2010, Lichtenstein *et al.* 2011). However, the precise mechanisms that regulate connexin trafficking to and from the plasma membrane and subsequently through to degradation are still being studied and debated. Posttranslational modifications of connexins are thought to contribute to the regulation of connexin function and trafficking. Connexin phosphorylation, which is known to regulate GJ channel closure and connexon membrane stability, has been studied intensively (Solan and Lampe 2009). More recently, other modifications have been identified such as hydroxylation, methylation, nitrosylation and acetylation, the last of which has been reported to affect connexin 43 localization in the heart (Locke and Harris 2009, Colussi *et al.* 2011).

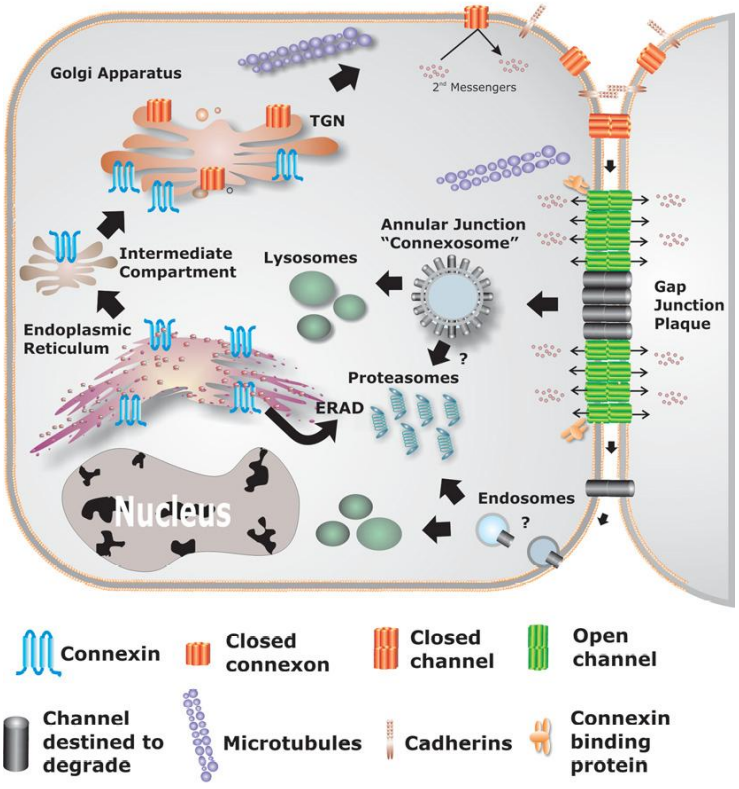

Figure 2. The lifecycle of connexins. Connexins are synthesized in the endoplasmic reticulum (ER) and oligomerize into connexons/hemichannels while trafficking through the ER, the ER-Golgi intermediate compartment, to the trans-Golgi network. Undocked connexons/hemichannels can open under certain conditions, allowing the exchange of small molecules between the intracellular and extracellular milieu. Connexons/hemichannels can also dock with neighboring hemichannels to form GJs. GJs internalize into annular junctions before degradation *via* the lysosomal or proteasomal pathway. ERAD = ER-associated degradation; TGN = trans-Golgi network; the question marks represent events that have not yet been clearly demonstrated. (Laird 2006)

1.3. Gap junctions

GJs are intercellular membrane channels that typically gather in groups of 10 to 10.000, socalled plaques, at the membrane surface. They arise from interaction of two opposing HCs, in turn consisting of six connexin proteins (Sohl and Willecke 2003). In vertebrates, only a few cell types do not form GJs in their fully differentiated state, including blood cells, spermatozoa, and skeletal muscle. Nevertheless, the progenitors of these cells do express GJs (Saez *et al.* 2003). GJs were originally identified in transmission electron micrographs (EM) as pentalaminar structures at points of close membrane apposition (Figueroa *et al.* 2004)(Figure 3). In the endothelium, long sections of well-defined GJs are commonly observed and form plaques of a few tents of a micrometer in diameter. GJs between endothelial cells and SMCs are called myoendothelial GJs (MEJ) (Figure 3) and have been extensively investigated as a direct route for communication between both cell types possibly influencing the contraction status of SMCs. In contrast to endothelium, gap junctional plaques in arterial smooth muscle have proven more difficult to visualize at the EM level, and often the junctional membranes are relatively widely separated even though the cells are dye coupled and electrically coupled. This had led to the suggestion that in vascular smooth muscle, individual gap junctional channels rather that GJ plaques are responsible for cell-cell communication (Figueroa *et al.* 2004).

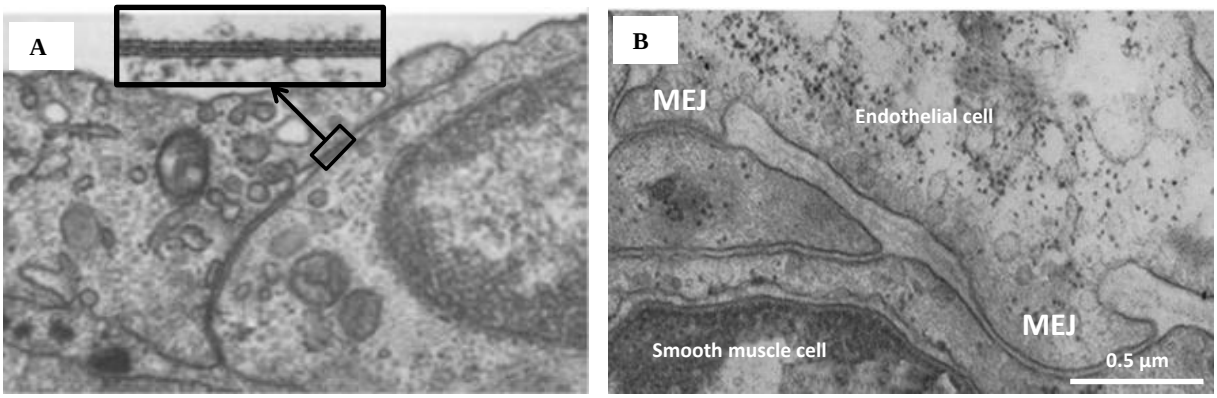

Figure 3. Gap junctions in the vascular wall. A. Transmission electron micrograph of the capillary endothelium. *Inset*: pentalaminar GJ at high magnification. B. Electron micrograph of an arteriolar myoendothelial gap junction (MEJ). (Figure adapted from Figueroa *et al.* 2004.)

GJs form the most direct cell-cell communication pathway, connecting the cytoplasm of adjacent cells. They have a central role in blood vessel physiology, helping in coordinating

the contraction of SMCs (Figueroa and Duling 2009), communicating electrical and Ca^{2+} signals in upstream direction *via* the endothelial layer (Dora and Duling 1997, Dora 2010) and contributing to fine tuning of EC-SMC communication. GJs coordinate several other crucial events such as the synchronized contraction of the cardiac muscle for the rhythmic pumping of the heart (Dhein *et al.* 2010), embryonic growth (Houghton 2005), bone remodeling (Jiang *et al.* 2007), tissue differentiation (Cronier *et al.* 2003), cell death and cell growth (Decrock *et al.* 2009).

The flux of molecules through these channels, called gap junctional intercellular communication (GJIC), includes the passive diffusion of small (< 1-1.5 kDa) and hydrophilic molecules, such as ATP, glucose, IP_3, glutamate, cAMP, gluthatione, H_2O_2, prostaglandins and ions (Ca^{2+}, K^+, Na^+) (Alexander and Goldberg 2003, Saez *et al.* 2003). GJ channels, depending on cellular needs and conditions, alternate between "closed" and "open" conformations. These changes are dependent on and are regulated by various mechanisms including calcium concentration, pH (Wang *et al.* 1996), transjunctional potential (Valiunas *et al.* 1997), and protein phosphorylation (Peracchia 2004, de Vuyst *et al.* 2007). Increases in $[Ca^{2+}]_i$ inhibit GJIC, albeit that the actual levels of $[Ca^{2+}]_i$ elevation necessary to suppress GJs is rather variable (from nanomolar to micromolar) and seems to depend on connexin and cell type (Spray *et al.* 1982, Lazrak and Peracchia 1993). The open probability of GJs furthermore depends on the transjunctional voltage (i.e. over the GJ), with large potential differences between cells resulting in closure of GJs. In addition, changes in connexin phosphorylation also affect GJIC (Kwak and Jongsma 1996, Lampe and Lau 2004, Moreno and Lau 2007).

1.4. Connexin hemichannels

In addition to forming GJs, CxHCs may also reside in the plasma membrane as unapposed free HCs not incorporated into GJs. Unapposed free CxHCs, are normally closed but they may open under certain conditions, often conditions regarded as stressful to cells. Several studies have shown that CxHCs, when open, may mediate the release of ATP, prostaglandins, glutamate, and gluthatione (Evans *et al.* 2006). ATP release *via* CxHCs has been studied extensively since its release activates purinergic receptors on neighboring cells and forms the basis of cell to cell Ca^{2+} signal propagation under the form of intercellular Ca^{2+} waves

(Burnstock 2006, Leybaert and Sanderson 2012). Although GJs and CxHCs allow molecules/ions up to 1.5 kDa to pass across, they have been shown to exhibit distinct sensitivities to increases of intracellular Ca^{2+} ($[Ca^{2+}]_i$). GJs close at 500-2000 nM $[Ca^{2+}]_i$ while CxHCs open between 200-500 nM and close again with higher $[Ca^{2+}]_i$ (de Vuyst *et al.* 2006, De Vuyst *et al.* 2009, Wang *et al.* 2012). In addition to moderate elevation of $[Ca^{2+}]_i$, CxHCs also open in response to a reduction of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) (Thimm *et al.* 2005, de Vuyst *et al.* 2006, De Vuyst *et al.* 2009). Other signals or conditions that control the functional state of CxHCs include metabolic inhibition (Contreras *et al.* 2002, Contreras *et al.* 2004), osmotic changes (Quist *et al.* 2000), membrane depolarization (Retamal *et al.* 2007), mechanical stimulation (Gomes *et al.* 2005) and oxidative (Ramachandran *et al.* 2007) and ischemic stress (Shintani-Ishida *et al.* 2007). CxHCs not only respond to cytoplasmic Ca^{2+}, but also determine cytoplasmic Ca^{2+}, as a Ca^{2+} entry pathway, suggesting a possible contribution to the cellular Ca^{2+} homeostasis and signaling. Furthermore, HCs, together with GJs, play a role in cell survival and cell death (see further in section *'connexin hemichannels and cell death'*). The separate role of CxHCs and GJ channels in physiology and pathology can be determined by the use of synthetic peptides that mimic short sequences in the connexin subunit (see section *'connexin mimetic peptides'*) (Evans *et al.* 2006).

1.5. Channel independent functions of connexin proteins

In addition to their multiple roles *via* exchange of molecules, whether through GJs or HCs, connexins by themselves also play various roles independent of their channel-forming properties. Such connexin functions are mediated through their multiple interacting partners and subsequently lead to the modulation of gene expression resulting in a wide range of effects (Jiang and Gu 2005).

Tumor suppressive properties of connexins has been shown for various connexins (e.g. Cx26 and Cx32), which suppressed growth, invasion, and metastasis of renal cell carcinoma cell lines, and this effect was mediated through various modulators including Src, tight junction proteins, VEGF and others (Sato *et al.* 2007). In addition, controlled expression of Cx43 prevented cell growth *via* the association of its C-terminal domain with proteins such as ZO-1 and c-Src (Moorby and Patel 2001). Multiple studies have shown that alterations of connexin

expression, whether through over-expression or deletion, lead to changes in gene expression in multiple pathways and cellular functions, including transcription, metabolism, cell/cell and cell/extracellular matrix adhesion, cellular signaling, transport, and cell cycle and division (Iacobas *et al.* 2004).

1.6. Connexin mimetic peptides

Peptides that correspond to specific short connexin sequences that block the operation of GJs and CxHCs, called connexin mimetic peptides, have been applied to study the structure and function of both channels (Locke and Harris 2009). The development and application of short peptides mimicking sequences in various protein domains of connexins, especially Cx43, initially focused on the two extracellular loops; recently peptides corresponding to sequences on the intracellular loop and carboxyl tail have been demonstrated to influence CxHC function. Warner *et al.* (1995) pinpointed motifs that included short sequence motifs, SHVR in extracellular loop 1 and SRPTEK in extracellular loop 2, as likely potent peptides for use in disrupting cell communication (Warner *et al.* 1995). These motifs form the basis of Gap26 (sequence: VCYDKSFPISHVR) and Gap27 (sequence: SRPTEKTIFII) mimetic peptides and their close homologues (Figure 4). The primary action of Gap26 and Gap27 was likely to be on CxHCs prior to blockage of GJs. Gap27 has been demonstrated to inhibit CxHC-mediated ATP release and dye uptake upon short (minutes) exposure (Braet *et al.* 2003), while longer exposures (hours) are generally needed to additionally inhibit GJs (Braet *et al.* 2003, Decrock *et al.* 2009). This was further illustrated by a voltage-clamp approach in nonconfluent and confluent HeLa cells expressing Cx43. Gap26 inhibited macroscopic currents through CxHCs in 2-3 min. In contrast electrical coupling in cell pairs was delayed and completely inhibited after 30 min or more (Desplantez *et al.* 2012). The extended time course of action on gap junctional electrical coupling suggests that longer diffusion pathways into plasma membrane junctional domains lead ultimately to blockage of gap junctional coupling. A complementary study by Wang *et al.* examined the mechanism of action of Gap26 and Gap27 in HeLa cells transfected with Cx43 and in pig ventricular myocytes endogenously expressing Cx43 using a voltage-clamp approach (Wang *et al.* 2012). Both mimetic peptides inhibited Cx43HC unitary currents within minutes. The precise protein domains to which Gap26 and Gap27 attach and any conformational changes induced in

connexin channels remain to be determined. HC dye uptake and dye release in Cx37 and Cx43 expressing rat brain endothelial cells exposed to bradykinin was rapidly (within minutes) inhibited by Gap27 (De Bock *et al.* 2011). A growing number of mimetic peptides derived from the cytoplasmic loop and C-terminus that act on CxHCs and GJs arise. Many of these peptides are not able to cross the plasma membrane and need to be attached first to "Trojan" cell-penetrating peptides that contain a membrane-translocation motif (Jarver and Langel 2006). Two of those 'newer' peptides are TAT-CT9 (sequence: YGRKKRRQRRRSRPRPDDLEI), which corresponds to the last 9 amino acids of Cx43 and TAT-L2 (sequence: YGRKKRRQRRRDGANVDMHLKQIEIKKFKYGIEEHGK), which correspond to the cytoplasmic loop (Figure 4). TAT-CT9 prevented inhibition of Cx43-hemichannel activity by high micromolar $[Ca^{2+}]_i$. This peptide prevented bradykinin-induced $[Ca^{2+}]_i$ oscillations in MDCK cells expressing Cx43 and other connexins (De Bock *et al.* 2012). TAT-L2 inhibited Cx43-hemichannel responses likely by binding to the last 9 or 10 amino acids of the C-terminal (CT) tail , thereby disrupting the intramolecular interaction between the CT tail and the endogenous L2 region (Ponsaerts *et al.* 2010). Other CT tail interaction sites may be involved as well. TAT-L2 had no inhibitory effect on GJs, it even favored the open state of Cx43 GJ channels and prevented closure of GJs (Delmar *et al.* 2004, Seki *et al.* 2004). The effects of connexin mimetic peptides on vascular tone will be discussed in section 3.4.

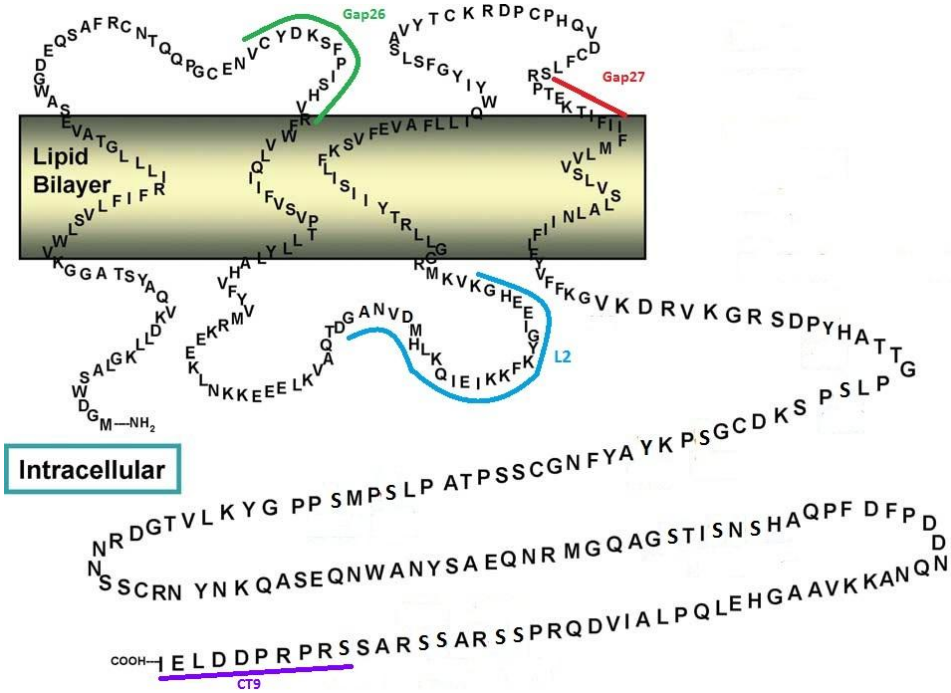

Figure 4. Connexin mimetic peptides for Cx43. The figure represents the peptides which mimic sequences of the extracellular loops (Gap26 and Gap27), cytoplasmic loop (L2) or carboxyl tail (CT9). (Figure adapted from Lampe *et al.* 2004)

1.7. Pannexins

Besides connexins, a new family of GJ-related channels has been identified, the pannexins (Panx) (Baranova *et al.* 2004, Scemes *et al.* 2009). Three pannexin isoforms have been described in rodents and human: Panx1, Panx2 and Panx3. They share a similar topology with connexins but have a different sequence homology. These channels primarily act as ATP-permeable HCs rather than GJs (Dahl and Locovei 2006). Among the pannexins, Panx1 has been best documented in terms of its HC function as a passageway for ions, metabolites and signaling molecules with a MW below 1 kDa, for example, ATP (D'Hondt *et al.* 2011). Panx1-mediated ATP release (Bao *et al.* 2004) has been shown to serve as a key physiological signal for intercellular signaling as well as a signal to promote cell death (D'Hondt *et al.* 2011). Pannexins have been recently identified as the channel mediating the “find me” signal released by apoptotic cells to recruit phagocytes in order to clear the dying cells (Chekeni *et al.* 2010). In the vasculature, a recent study reported a role of Panx1 in the contraction of resistance arteries where phenylepinephrine activates the release of vasoactive purines, such as ATP, through Panx1 channels (Billaud *et al.* 2011). Panx1 channel opening can also be detrimental, contributing to cell death and seizures under ischemic or epileptic conditions

(Thompson *et al.* 2008, Santiago *et al.* 2011), and even facilitating HIV-1 viral infection (Seror *et al.* 2011). Panx2 has been shown to be involved in differentiation of neurons (Swayne *et al.* 2010) and its channel function can contribute to ischemic brain damage (Bargiotas *et al.* 2011). Panx3 has been reported to play a role in differentiation of chondrocytes (Iwamoto *et al.* 2010) and osteoblasts (Ishikawa *et al.* 2011) while playing a key role in the maturation and transport of sperm (Turmel *et al.* 2011). Panx1 can be opened by mechanical stress (Bao *et al.* 2004), membrane depolarization (Bruzzone *et al.* 2005), increases in $[Ca^{2+}]_i$ (Locovei *et al.* 2006), and activation of purinergic receptors, ATP and agonists (Locovei *et al.* 2006, D'Hondt *et al.* 2009). Pannexins are blocked by pannexin mimetic peptides and certain connexin-based channel inhibitors such as carbenoxolone, flufenamic acid and mefloquine (Bruzzone *et al.* 2005, Pelegrin and Surprenant 2006, D'Hondt *et al.* 2009).

2. Connexins in the cardiovascular system

In the vasculature Cx37, Cx40, Cx43 and Cx45 are present (Spray *et al.* 2011). The expression of connexins in the vessel wall is not uniform and seems to vary with vessel size, vascular territory and species. In most cases Cx45 is observed only in SMCs. Cx37 is typically confined to ECs, although it has also been detected in SMCs. In contrast, Cx40 and Cx43 may be expressed in both cell types, but Cx40 is predominantly located in endothelial cells and Cx43 is the most prominent connexin type found in SMCs (Figueroa and Duling 2009). The importance of connexins in the vasculature is highlighted by the fact that knock-out of several connexins disturbs vascular tone and development. Knockout of Cx40 is associated with irregular arteriolar vasomotion, impaired conduction of dilatory signals and hypertension (Kirchhoff *et al.* 1998, Simon *et al.* 1998, de Wit *et al.* 2000, de Wit *et al.* 2003). In Cx37 knockout mice K^+-induced conducted vasoconstriction was significantly attenuated (McKinnon *et al.* 2006). The loss of Cx37 was not lethal (Simon and McWhorter 2002) and did not affect the conduction of vasodilation by acetylcholine in arterioles or gave rise to a cardiovascular phenotype (Schmidt *et al.* 2008). Research demonstrated an association of a polymorphism in the Cx37 gene, a protein normally expressed in endothelial cells but also found in monocytes and macrophages, with arterial stenosis and myocardial infarction in humans (Boerma *et al.* 1999, Yeh *et al.* 2003, Listi *et al.* 2005, Wong *et al.* 2006). Cx43 deletions modify the expression of many genes in the differentiation and function of vascular cells, with alterations in the coronary artery development (Walker *et al.* 2005). Cx43 plays a role in the looping of the ascending limb of the heart tube and the development of the right ventricle and the outflow tract, and Cx43-knockout mice die at birth of severe cardiac malformations (Reaume *et al.* 1995). Loss of Cx43 specifically confined to the endothelium was found to cause hypotension and bradycardia (Liao *et al.* 2001). In another study, lack of Cx43 in the endothelium had no effect on the blood pressure (Theis *et al.* 2001). Cx45 is expressed in the smooth muscle layer of vessels and, to a lesser extent, also in the endothelium at least at early stages of development (Kruger *et al.* 2000). Knock-out of Cx45 blocks vascular development after the normal initiation of vessel formation, and impairs the differentiation of SMCs. Cx45-deficient mice die during embryonic development exhibiting striking abnormalities of vascular development (Kruger *et al.* 2000). Figure 5

shows a schematic illustration of the distribution pattern of connexin expression in the cardiovascular system.

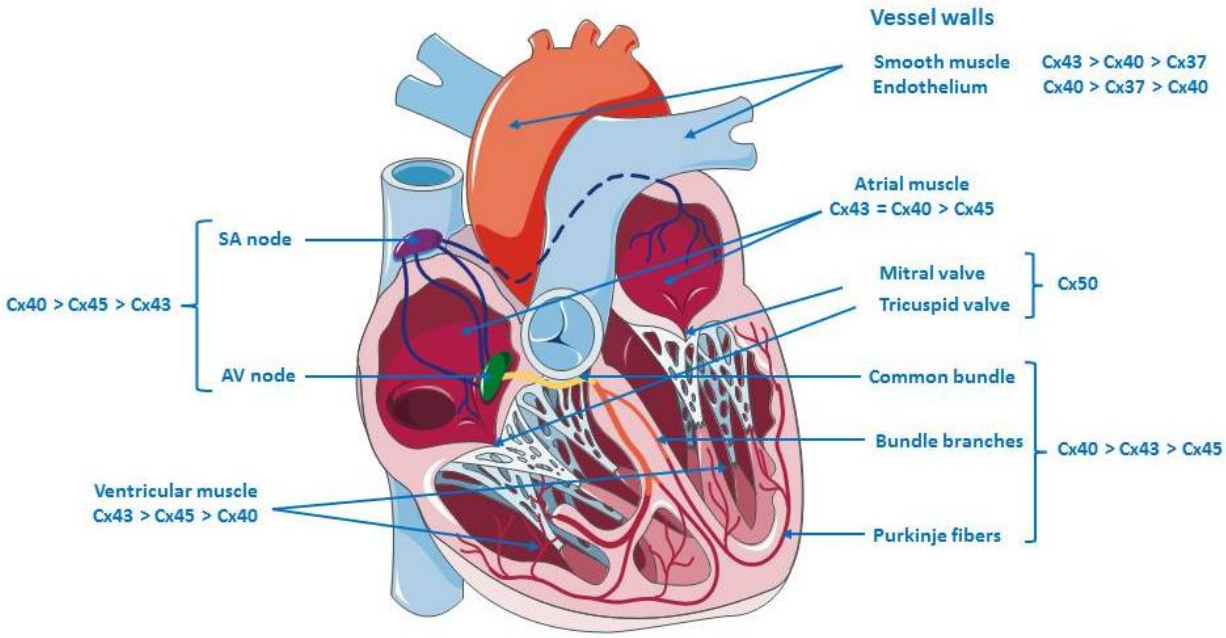

Figure 5. Schematic diagram illustrating general pattern of distribution and relative abundance of connexin types expressed in different regions of the mammalian heart and vessel wall. (Figure adapted from Spray et al. 2011)

The expression of connexins has been reported to be altered in several pathologies associated with vascular complications, such as hypertension (Rummery and Hill 2004, Figueroa *et al.* 2006), atherosclerosis (Kwak *et al.* 2002, Chadjichristos and Kwak 2007) and diabetes (Inoguchi *et al.* 1995, Zhang and Hill 2005). For example, endothelial Cx43 was upregulated in the initial phase of atherosclerosis (Gabriels and Paul 1998) and further progression of the disease downregulated endothelial Cx37 and upregulated Cx37 in macrophages recruited to the lesions (Kwak *et al.* 2002). Recently a link has been found between NO signaling and connexins and it is clear that in vascular diseases, such as atherosclerosis, hypertension and diabetes both NO signaling and connexin expression and/or function are altered (Looft-Wilson *et al.* 2011). NO affects vascular GJIC *in vitro* and *in vivo*, with both enhancement and inhibition of communication reported depending on the connexins involved and the cellular location within the vessel (McKinnon *et al.* 2009, Looft-Wilson *et al.* 2011, Straub *et al.* 2011). There is new evidence that not only nitric oxide

interacts with connexins, but the enzyme endothelial NO synthase (eNOS) does as well. In endothelial cells, eNOS can exist as a complex with at least two connexins (Cx40 and Cx37) and this interaction affects eNOS function and expression at the membrane (Alonso *et al.* 2010). It is also known that loss of eNOS affects gap junctional communication in arteries (Rodenwaldt *et al.* 2007).

3. Connexins and calcium signaling

3.1. Introduction

Many cellular functions are regulated by changes in $[Ca^{2+}]_i$. This generates Ca^{2+} signals that are highly organized in both time and space. It is well accepted that modulation of $[Ca^{2+}]_i$ is a critical determinant of vascular smooth muscle tone. Several Ca^{2+} signals in vascular preparations are described based on their spatio-temporal organization. *Ca^{2+} sparks* are highly localized, transient increases in $[Ca^{2+}]_i$, which occur in both isolated SMCs and intact arteries and are only observed at rest, or at very low level of stimulation (Dupont *et al.* 2007). At higher stimulation levels, *Ca^{2+} oscillations* appear, *i.e.* transient rises in $[Ca^{2+}]_i$, that start from a specific region of the cell and are propagated along its length in a wave-like manner. Ca^{2+} oscillations may allow Ca^{2+} to function as a messenger while limiting the toxic effects of a sustained elevation in $[Ca^{2+}]_i$ (Savineau and Marthan 2000)**.** Two main types of Ca^{2+} oscillations have been reported: asynchronous Ca^{2+} oscillations, underlying tonic contractions of arteries and veins, and synchronous Ca^{2+} oscillations signaling vasomotion, which are spontaneous, rhythmical contractions, in some resistance arteries (Dai *et al.* 2010). Asynchronous Ca^{2+} oscillations are caused by regenerative Ca^{2+} release from the sarcoplasmic reticulum (SR) and maintained by refilling of the SR by several channels and exchange pumps in the plasma membrane (see further). Synchronized Ca^{2+} oscillations are also paced by periodic Ca^{2+} release from the SR, which however, does not directly activate myofilaments, but serves to activate Ca^{2+} dependent Cl^- channels, which depolarize the plasma membrane and activate L-type Ca^{2+} channels in electrically coupled SMCs (Jacobsen *et al.* 2007).

3.2. Asynchronous Ca^{2+} oscillations

In 1994, asynchronous Ca^{2+} oscillations were first described by Iino *et al.* (Iino *et al.* 1994); Ca^{2+} oscillations in individual SMCs of rat tail arteries were induced by norepinephrine. These oscillations occurred in the form of repetitive intracellular Ca^{2+} waves that propagated along the longitudinal axis of the ribbon-shaped SMCs. The Ca^{2+} waves did not spread intercellularly and were not synchronized between neighbouring SMCs. Since then similar asynchronous Ca^{2+} oscillations were found in a large set of other vessel types in response to

a variety of agonists such as phenylephrine, norepinephrine, angiotensin II, UTP or high K^+ solutions (Ruehlmann *et al.* 2000, Lee *et al.* 2001, Mauban *et al.* 2001, Peng *et al.* 2001). The concentration of the agonist is the main factor that modulates the pattern of Ca^{2+} oscillations in SMCs (Ruehlmann *et al.* 2000). But in some cells, the oscillation pattern seems to be independent of the agonist concentration, but the percentage of cells oscillating in response to agonist stimulation does depend on the agonist concentration (Savineau and Marthan 2000).

Calcium oscillations are initiated by binding of the extracellular agonist to its receptor and this leads, *via* a G-protein activation cascade, to phospholipase C (PLC) activation. PLC catalyzes the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 binds to receptors located on the SR surface (Figure 6). The Ca^{2+} release initiated by this binding can trigger successive cycles of activation/inhibition of the IP_3 receptor resulting in sustained Ca^{2+} oscillations.
A bimodal regulation of IP_3-induced Ca^{2+} release is mostly accepted as the basis of Ca^{2+} oscillations (Iino 2000). The IP_3 receptor channel is highly regulated by the cytoplasmic Ca^{2+} concentration. Indeed, up to a certain level $[Ca^{2+}]_i$ promotes Ca^{2+}-induced Ca^{2+} release (CICR) whereas $[Ca^{2+}]_i$ exceeding this threshold provides a negative feedback on IP_3-induced Ca^{2+} release (IICR), initiating the declining phase of the Ca^{2+} spike (Ilyin and Parker 1994). The declining phase of the wave can be seen as a combination of a decrease of the Ca^{2+} release and the active removal of Ca^{2+} from the cytosol depends on the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Mumtaz *et al.* 2011), that pumps some of the Ca^{2+} back into the ER, and the plasma membrane Ca^{2+}-ATPase (PMCA) or Na^+/Ca^{2+} exchanger (Dora 2001, Lee *et al.* 2001), that pumps Ca^{2+} out of the cell (Berridge 2008). In such a manner, Ca^{2+} release can trigger successive cycles of IP_3 receptor activation-inhibition resulting in Ca^{2+} oscillations. To entrain this mechanism, Ca^{2+} entry *via* several ways (Figure 6) such as L-type voltage-operated Ca^{2+} channels (VDCCs), TRP channels, ATP sensitive P2X receptors and the Na^+/Ca^{2+} exchanger in the reverse mode, is needed (Guibert *et al.* 1997, Hyvelin *et al.* 1998, Lee *et al.* 2001, Syyong *et al.* 2009).

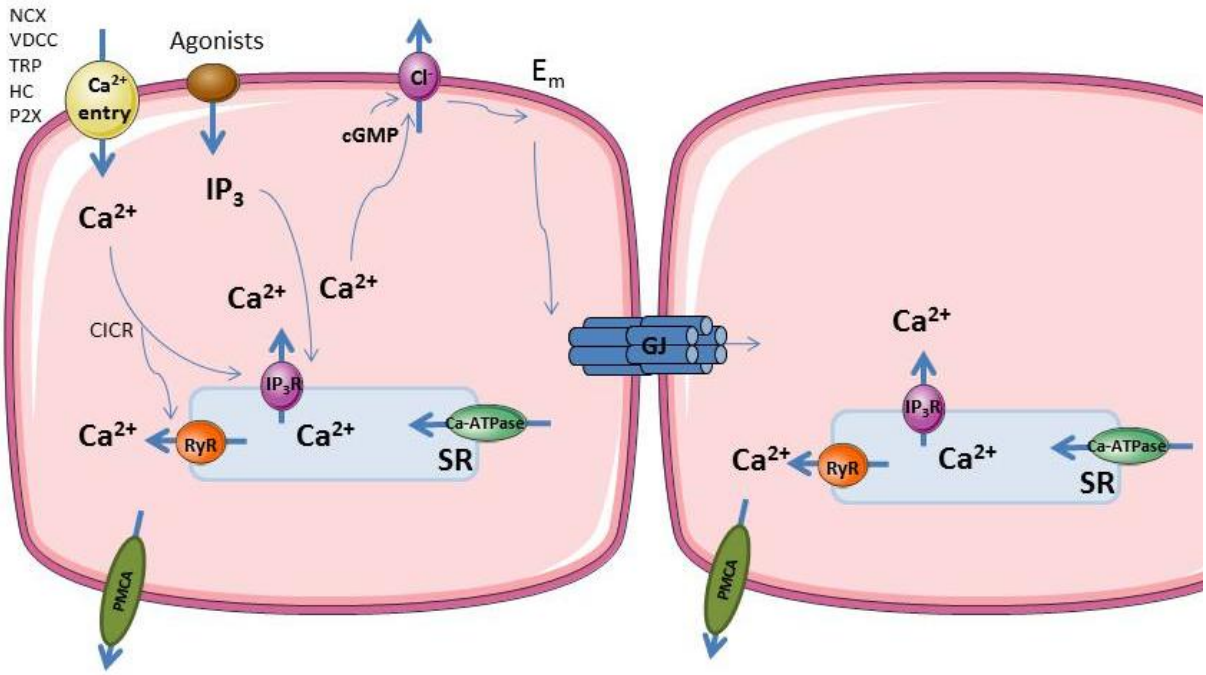

Figure 6. Schematic diagram of Ca^{2+} signaling in vascular smooth muscle cells. Ca^{2+} oscillations are initiated by agonist binding to its receptor. This leads to the formation of IP_3. IP_3 binds to its receptor and a subsequent release of Ca^{2+} can trigger successive cycles of activation/inhibition of IP_3 receptor resulting in Ca^{2+} oscillations. To entrain this mechanism, Ca^{2+} entry *via* voltage-dependent Ca^{2+} channels (VDCC), Na^{+}–Ca^{2+} exchanger in reverse mode (NCX), hemichannels (HC), transient receptor potential ion channels (TRP) and ATP-sensitive P2X receptors (P2X) is needed. This Ca^{2+} entry promotes Ca^{2+}-induced Ca^{2+} release (CICR) *via* IP_3R or RyR up to a certain $[Ca^{2+}]_i$ level.When $[Ca^{2+}]_i$ exceeds this threshold a negative feedback mechanism initiates the declining phase of the Ca^{2+} wave by a decrease in Ca^{2+} release and active removal of Ca^{2+} from the cytosol. Removal of Ca^{2+} from the cytosol depends on the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that pumps Ca^{2+} into the sarcoplasmic reticulum (SR) and the plasma membrane Ca^{2+}-ATPase (PMCA) or NCX exchanger to pump Ca^{2+} outside the cell. Ca^{2+} oscillations can trigger cGMP-dependent Cl^{-} channels to open and this leads to a depolarization of the membrane potential (E_m). This depolarizing current can pass through gap junctions (GJ) and this will start the vasomotion mechanism.

GJs are known to influence the frequency of the Ca^{2+} oscillations, the number of cells displaying Ca^{2+} oscillations and the synchronization of the Ca^{2+} oscillations (Clair *et al.* 2001, Fanchaouy *et al.* 2005). Recently, the picture is further complicated by the possible contribution of CxHCs in the process of Ca^{2+} oscillations (Figure 7). Release of Ca^{2+} *via* the IP_3 receptor can influence the opening of CxHCs and this is followed by HC-mediated Ca^{2+} entry. Opening of CxHCs can also lead to the release of ATP. Ca^{2+}-triggered ATP release *via* HCs followed by autocrine action of ATP on cell-surface receptors and subsequent regeneration of the cytoplasmic Ca^{2+} signal constitutes a putative signaling loop necessary for these oscillations (Kawano *et al.* 2006, Verma *et al.* 2009). De Bock *et al.* demonstrated recently

that bradykinin, a peptide that activates the PLC-IP_3-pathway, triggers Ca^{2+} oscillations involving CxHCs (De Bock *et al.* 2011).

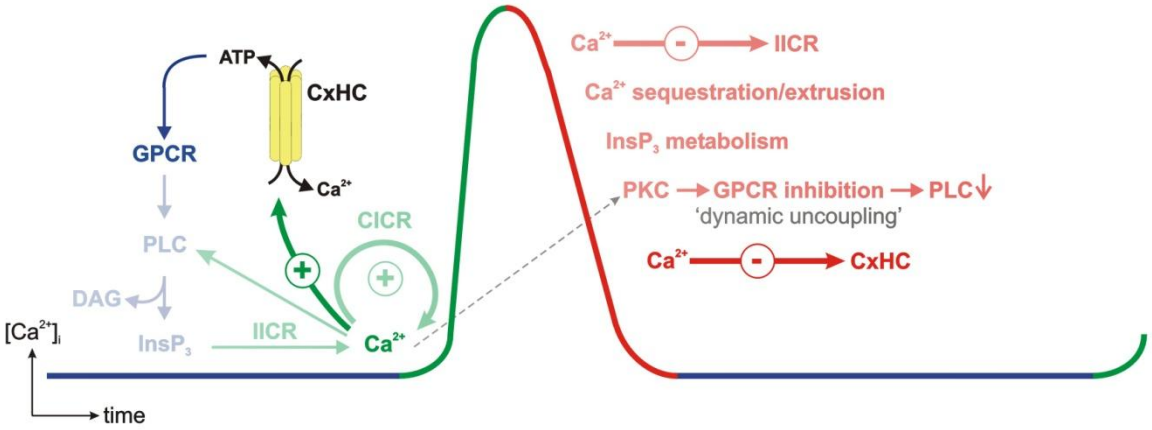

Figure 7. Diagram of basic mechanisms involved in generating an oscillatory Ca^{2+} spike and the contribution of connexin hemichannels in IP_3-based Ca^{2+} oscillations. Ligand binding of a G protein coupled receptor (GPCR) generates IP_3 which leads to IP_3-induced Ca^{2+} release (IICR) that is further amplified by CICR (positive feedback) and Ca^{2+} activation of PLC, producing the rising phase of the Ca^{2+} spike. Elevated $[Ca^{2+}]_i$ will inactivate IICR (negative feedback) and stimulate Ca^{2+} sequestration and extrusion. Metabolic conversion of IP_3 to IP_2 and IP_4 will lower the IP_3 concentration. Ca^{2+}, together with diacylglycerol (DAG), will also activate protein kinase C (PKC) (dotted line) suppressing GPCRs and PLC activity. Once all OFF mechanisms have restored $[Ca^{2+}]_i$ to the resting level, the cycle is ready for a next Ca^{2+} spike.
Moreover, connexin hemichannel opening by moderate $[Ca^{2+}]_i$ elevation results in Ca^{2+} entry, accompanied by ATP release. ATP acts on GPCRs at the cell surface thereby enhancing IP_3 production, while Ca^{2+} entry *via* hemichannels contributes to the rising phase of the Ca^{2+} spike. When $[Ca^{2+}]_i$ rises above 500 nM, connexin hemichannels close and this contributes to the restoration of resting $[Ca^{2+}]_i$. (De Bock *et al.* 2012)

Asynchronous Ca^{2+} oscillations induced by extracellular agonists are always mediated by IP_3 (Aalkjaer and Nilsson 2005); however, other messengers may also be involved: cyclic ADP ribose (cADPR) that activates the ryanodine receptor (RyR) channel and nicotinic acid adenine dinucleotide phosphate (NAADP) that activates two-pore channels (TPC). The periodic release of Ca^{2+} from the ER (*via* IP_3R, RyR, or TPC) is important to induce synchronized Ca^{2+} oscillations and subsequent vasomotion.

3.3. Vasomotion / Synchronous Ca^{2+} oscillations

In some blood vessels, a specific tone is maintained by the spatial averaging of asynchronous Ca^{2+} oscillations. However, in some vessels Ca^{2+} oscillations in groups of cells are synchronized and this results in the pulsatile contractions known as vasomotion (Haddock and Hill 2005). Rhythmical contractions are generated in many different types of smooth muscle, from the gastro-intestinal tract, urinary tract and lymphatic vessels through to arteries and veins. In blood vessels, this activity known as vasomotion occurs in small resistance vessels of the microcirculation, as well as in larger arteries both *in vivo* and *in vitro*. Rhythmicity in vascular smooth muscle is apparently synchronous over considerable lengths of arteries. Vasomotion is thus expected to increase flow as its amplitude increases, in turn resulting in a decrease in vascular resistance. In this case vasomotion may be seen to be beneficial and its up-regulation during pathological conditions, such as hypertension, may be considered to be protective (Haddock and Hill 2005, Chen *et al.* 2010). Studies on both animals and humans indicate a tight coupling between blood pressure level and the ability of vessels to oscillate (Hollenberg and Sandor 1984, Chen *et al.* 2010). Pathologies such as diabetes and ischemia are associated with altered prevalence of vasomotion, and there is evidence that vasomotion has a protective role during ischemia. The latter suggestion is strongly supported by the findings that vasomotion enhances dialysis of the extravascular fluid (Lefrandt *et al.* 2003, Sakurai and Terui 2006). This might relate to the observation that impaired vasomotion favors the development of skin lesions and hypertension in patients with type 2-diabetes (Broegger *et al.* 2011).

Vasomotion requires both a mechanism which can initiate the oscillation and a mechanism for the synchronization of the SMCs in the vascular wall. It has been proposed that oscillatory release of Ca^{2+} from the SR, induced or modulated by neurotransmitters or hormones, is responsible for initiating the oscillation. This hypothesis is supported by several studies showing that vasomotion could be abolished by inhibiting the release of Ca^{2+} from SR. Those periodic releases of Ca^{2+} cause a membrane depolarization of the cell. This depolarization can spread to neighboring cells by current flow through the GJs to provide a synchronization mechanism (Nilsson *et al.* 2003, Berridge 2008). The membrane depolarization will activate voltage-dependent Ca^{2+} channels in neighboring cells to provide a Ca^{2+} pulse that will help to iniate their Ca^{2+} transients thus bringing them into phase with

each other. Figure 8 shows the model for the generation of vasomotion in rat mesenteric small arteries.

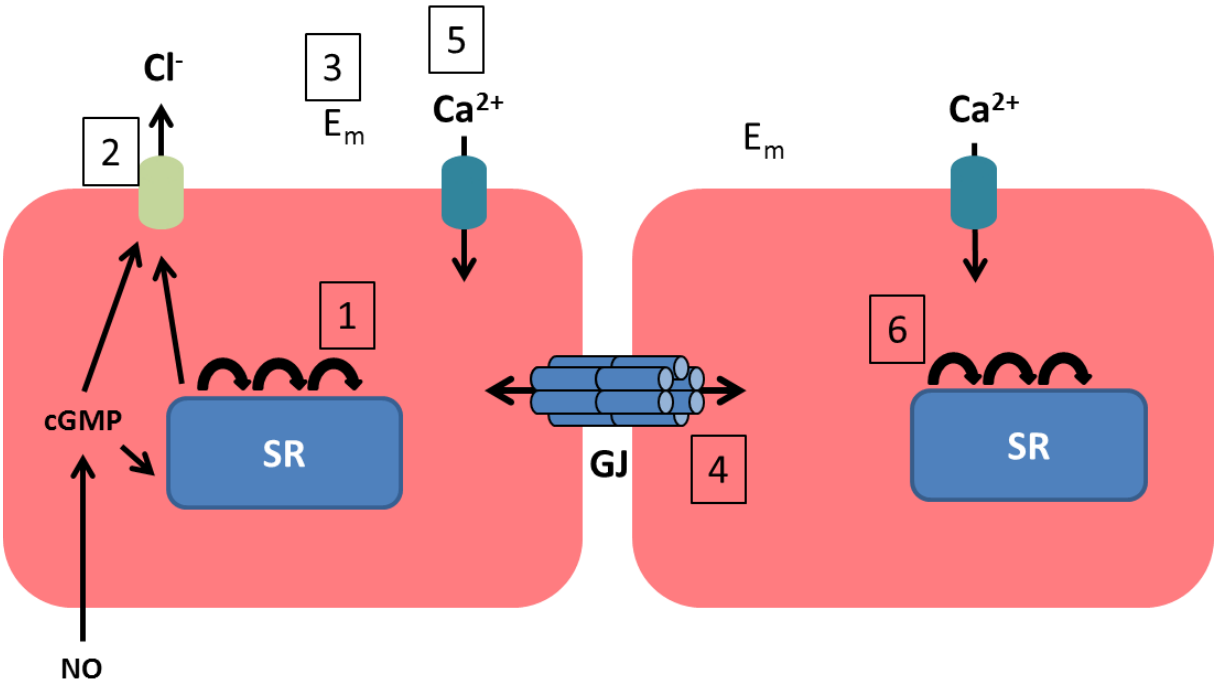

Figure 8. Model for the generation of vasomotion in rat small mesenteric arteries. 1. The basic oscillator is the sarcoplasmic reticulum (SR), which releases Ca^{2+} leading to regenerative Ca^{2+} waves traversing the cell. 2. In the presence of cGMP, this Ca^{2+} is able to activate a chloride channel (Cl^-) in the plasma membrane. 3. If this current is activated in a sufficient number of cells at the same time, cells will depolarize. 4. This depolarization can spread to neighboring cells *via* GJs. 5. Depolarization will cause Ca^{2+} influx through L-type Ca^{2+} channels and contraction. 6. This Ca^{2+} influx will also release Ca^{2+} in quiescent cells and thus resetting their intracellular oscillators.

Other types of oscillations, either based solely on interactions of ion currents in the sarcolemma or based on oscillations of the glycolytic pathway and consequently the Na-K-pump, have also been suggested and could play a role under some conditions, although these pathways are probably less frequent (Aalkjaer and Nilsson 2005).

Vasomotion has been shown in many studies of isolated arteries to be endothelium dependent and several reports support that endothelial removal prevents vasomotion (Peng *et al.* 2001, Sell *et al.* 2002, Rahman *et al.* 2005, Boedtkjer *et al.* 2008)). In rat mesenteric arteries, removal of the endothelium desynchronizes $[Ca^{2+}]_i$ oscillations and abolishes vasomotion (Peng *et al.* 2001) and this has been proposed to be due to a loss of NO and the

subsequent activation of a cGMP-dependent depolarizing current in the SMCs. Addition of cell-permeable cGMP analogues was shown to restore vasomotion in endothelium-denuded tissues (Jackson *et al.* 1991, Gustafsson 1993). The synchronizing effect of NO is complex. NO can activate directly or through sGC/cGMP depolarizing and hyperpolarizing currents that can affect synchronization (Kapela *et al.* 2012). However, in the same artery, and in other vessels, NO has been reported to have an inhibitory effect on vasomotion and under these conditions it is unlikely to be responsible for initiating vasomotion (Omote *et al.* 1992, Hill *et al.* 1999, Sell *et al.* 2002, Mauban and Wier 2004). Seppey *et al.* showed that it is possible to obtain vasomotion in rat small mesenteric strips in the presence and in the absence of an intact endothelium. The range of agonist concentrations leading to vasomotion seems to greatly differ between these two cases. Three times more phenylephrine was needed to induce vasomotion in arterial strips with an intact endothelium to compensate the decreasing contractility by the endothelium-derived factors (Seppey *et al.* 2008). Next to NO, EDHF has been suggested to initiate vasomotion (Okazaki *et al.* 2003, Mauban and Wier 2004). Vasomotion is abolished and only asynchronous $[Ca^{2+}]_i$ oscillations can be detected in SMCs following inhibition of EDHF with the K_{Ca} channel blockers apamin and charybdotoxin (Mauban and Wier 2004). Since Ca^{2+} and IP_3 can move through MEJ from SMCs into ECs (Schuster *et al.* 2001, Lamboley *et al.* 2005, Isakson *et al.* 2007), it has been proposed that these molecules could activate K_{Ca} channels in the endothelium. The resulting hyperpolarization would be electrotonically propagated back into the SMCs.

3.4. Interfering with connexin channels modulates the vascular tone

A number of studies have demonstrated that GJ uncouplers such as heptanol, 18α-glycyrrhetinic acid, carbenoxolone and connexin mimetic peptides abolish synchronized contractions (Jackson *et al.* 1991, Chaytor *et al.* 1997, Hill *et al.* 1999, Matchkov *et al.* 2006), synchronized waves (Sell *et al.* 2002, Martin *et al.* 2005, Matchkov *et al.* 2006) and oscillations in membrane potential (Hill *et al.* 1999). However, a growing body of evidence suggests that many of these compounds have additional non-specific effects on targets other than GJs (Spray and Burt 1990, Chaytor *et al.* 1997, Tare *et al.* 2002, Matchkov *et al.* 2004) and so the results of these studies must be interpreted with caution.

Connexin mimetic peptides, i.e. peptides that are identical with a short amino acid sequence on the connexin subunit, were initially designed to produce connexin-selective blockage of GJs, but have recently emerged as a tool to block CxHCs with little or no immediate effects on GJs (Evans *et al.* 2012). In most cases, a time window can be defined where HCs are inhibited (1 h exposure time for instance) without concurrent effects on GJs (Wang *et al.* 2012). Gap26 and gap27 peptides appear to act in a connexin-specific manner and have been widely applied to block GJs in the past. These peptides inhibit gap junctional transfer of fluorescent dyes (Chaytor *et al.* 1999), electrical coupling (Dora *et al.* 1999) and synchronized Ca^{2+} oscillations (Isakson and Duling 2005, Martin *et al.* 2005, Matchkov *et al.* 2006) in SMCs. The peptides also inhibit several cellular integrative processes mediated by Cxs and/or GJs, such as synchronized rhythmic activity of arteries (Chaytor *et al.* 1997, Griffith *et al.* 2004, Figueroa *et al.* 2006, Matchkov *et al.* 2006) and endothelial smooth-muscle interactions governing relaxation of blood vessels (Chaytor *et al.* 1999, Dora *et al.* 1999, De Vriese *et al.* 2002, Matchkov *et al.* 2006, Tang and Vanhoutte 2008, Lopez *et al.* 2009). The authors suggested a role for (myo)endothelial GJs in the regulation of vascular tone. Matchkov and others suggested that a peptide combination of ^{43}gap26, ^{40}gap27 and ^{37,43}gap27 suppressed vasomotion in rat mesenteric small arteries (Matchkov *et al.* 2006); an effect that was attributed to an interruption of intercellular communication *via* GJs.

Braet *et al.* were the first to report that connexin mimetic peptides also inhibit CxHCs (Braet *et al.* 2003, Braet *et al.* 2003). This observation was somehow expected, given the fact that the extracellular-loop sequences, with which the peptides are likely to interact, are freely available in the CxHC form. Both gap26 and gap27 blocked uptake of the reporter dye propidium iodide and ATP release in Cx43-expressing cells, and the effect became apparent after short incubations, typically in the order of 10-30 min. Moreover, Wang *et al.* showed in HeLa cell lines and pig ventricular cardiomyocytes that unitary HC currents were strongly inhibited by both peptides within minutes (Wang *et al.* 2012). For comparison, the effects of connexin mimetic peptides on GJs typically require longer incubations of the order of about 1 hour or more (Evans *et al.* 2006, Evans and Leybaert 2007).

Recently new connexin mimetic peptides were discovered by the hostlab in collaborative research work with the group of Geert Bultynck (KULeuven). These block Cx43 HCs, without interfering with gap junctional communication (Ponsaerts *et al.* 2010, Wang *et al.* 2013). Two peptides corresponding to the cytoplasmic loop of Cx43 (L2 peptide and Gap19, part of

the L2 sequence) were shown to inhibit CxHC mediated responses (Ca^{2+} waves and ATP release) (Ponsaerts *et al.* 2010) and HC unitary currents (Wang *et al.* 2013)

Lately pannexins were shown to be involved in the control of vasoconstriction. This was due to the release of purines through Panx1 channels on VSMCs triggered by phenylephrine stimulation and participates in the control of vascular tone by purinergic receptors (Billaud, Lohman *et al.* 2011).

4. Connexins and cell death

4.1. Introduction

In multicellular organisms, the maintenance of tissue homeostasis ultimately relies on the critical balance between cell growth and cell death. Abnormalities in the control of cell death contribute to a variety of cardiovascular diseases; for example, regulated cell death is involved in vessel remodeling during development or following injury, but deregulated death is implicated in pathologies such as atherosclerosis, aneurysm formation, restenosis after angioplasty/stenting, vascular graft infection, ischemic and dilated cardiomyopathies and infarction (Clarke *et al.* 2007). Cell death is characterized by a number of distinct mechanisms, including apoptosis, autophagy and necrosis. Apoptosis, necrosis and autophagy are defined on the basis of morphology, tissue location and the dependence of lysosomes.

a) Apoptosis

Apoptosis is an energy-dependent process characterized by rounding-up of the cell, reduction of cellular volume (pyknosis), minor changes in cytoplasmic chromatin condensation, nuclear fragmentation (karyorrhexis), plasma membrane blebbing (but maintenance of its integrity until the final stages of the process) and lysosomal degradation of apoptotic bodies by phagocytes (Elmore 2007, Kroemer *et al.* 2009, Orrenius *et al.* 2011). The efficient recognition of apoptotic cells by phagocytes requires rearrangement of the infrastructure and molecular composition of the plasma membrane in the dying cell. For example, alteration of the distribution of carbohydrates on the cell surface promotes preferential binding of macrophages to apoptotic cells. The loss of phospholipid asymmetry, resulting in the externalization of phosphatidylserine (PS), further facilitates the recognition of dying cells by macrophages. In fact, PS exposure on the apoptotic cell surface appears to be the most important “eat me” signal, although its binding to macrophages is usually not direct but mediated by a number of bridging molecules (Hanayama *et al.* 2004). However, it should be noted that under *in vitro* conditions, where phagocytic cells are normally absent, apoptotic cells and their fragments lyse in a process similar to necrosis. This is termed secondary necrosis (Orrenius *et al.* 2011). *In vivo* secondary necrosis occurs when massive apoptosis overwhelms the available scavenging capacity, or when the scavenger mechanism

is directly impaired, and may result in leakage of the cell contents with induction of tissue injury and inflammatory and autoimmune responses (Silva *et al.* 2008)

Two major apoptotic pathways can be distinguished, the mitochondria-mediated intrinsic cascade and the death receptor-mediated extrinsic pathway (Figure 9). Both rely on the proteolytic activity of an evolutionary conserved family of cysteine proteases – the caspases – which form the biochemical basis of the apoptotic phenotype (Earnshaw *et al.* 1999). They are responsible for the cleavage of a large number of cellular proteins including major cytoplasmic and nuclear elements. The extrinsic signaling pathway is triggered by the binding of an extracellular death ligand, such as Fas ligand, tumor necrosis factor α (TNFα) or TNF-related apoptosis-inducing ligand (TRAIL) to their corresponding receptors at the plasma membrane (Figure 9). This is followed by the formation of the death-inducing signaling complex (DISC) and resulted in the activation of pro-caspase-8 (Gump and Thorburn 2011). In type I cells, caspase-8 activates procaspase-3, which cleaves target proteins, leading to apoptosis. In type II cells, caspase-8 cleaves the pro-apoptotic B-cell lymphoma-2 (Bcl-2) protein Bid, which, in turn, induces the translocation, oligomerization, and insertion of the Bcl-2-associated X protein (Bax) and/or Bcl-2 antagonist/killer (Bak) into the mitochondrial outer membrane. This is followed by the release of several proteins from the mitochondrial intermembrane space, including cytochrome C (CytC), which forms a cytosolic apoptosome complex with Apaf-1 (apoptosis activating factor-1) and procaspase-9 in the presence of dATP. This results in the activation of procaspase-9, which triggers the caspase cascade by activation of procaspase-3 (Orrenius *et al.* 2011).

In contrast, the intrinsic signaling pathway is mediated by mitochondria and involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals directly acting on targets within the cell (Figure 9). It is regulated by the Bcl-2 family of pro- and anti-apoptotic proteins, inhibitor of apoptosis proteins (IAPs) (inhibition of caspases), second mitochondrial activator of caspases (Smac), and Omi (negative regulation of IAPs). The intrinsic pathway may also operate *via* caspase-independent mechanisms, which involve the release from mitochondria and translocation to the nucleus of at least two proteins, apoptosis inducing factor (AIF) and endonuclease G (EndoG) (Orrenius *et al.* 2011).

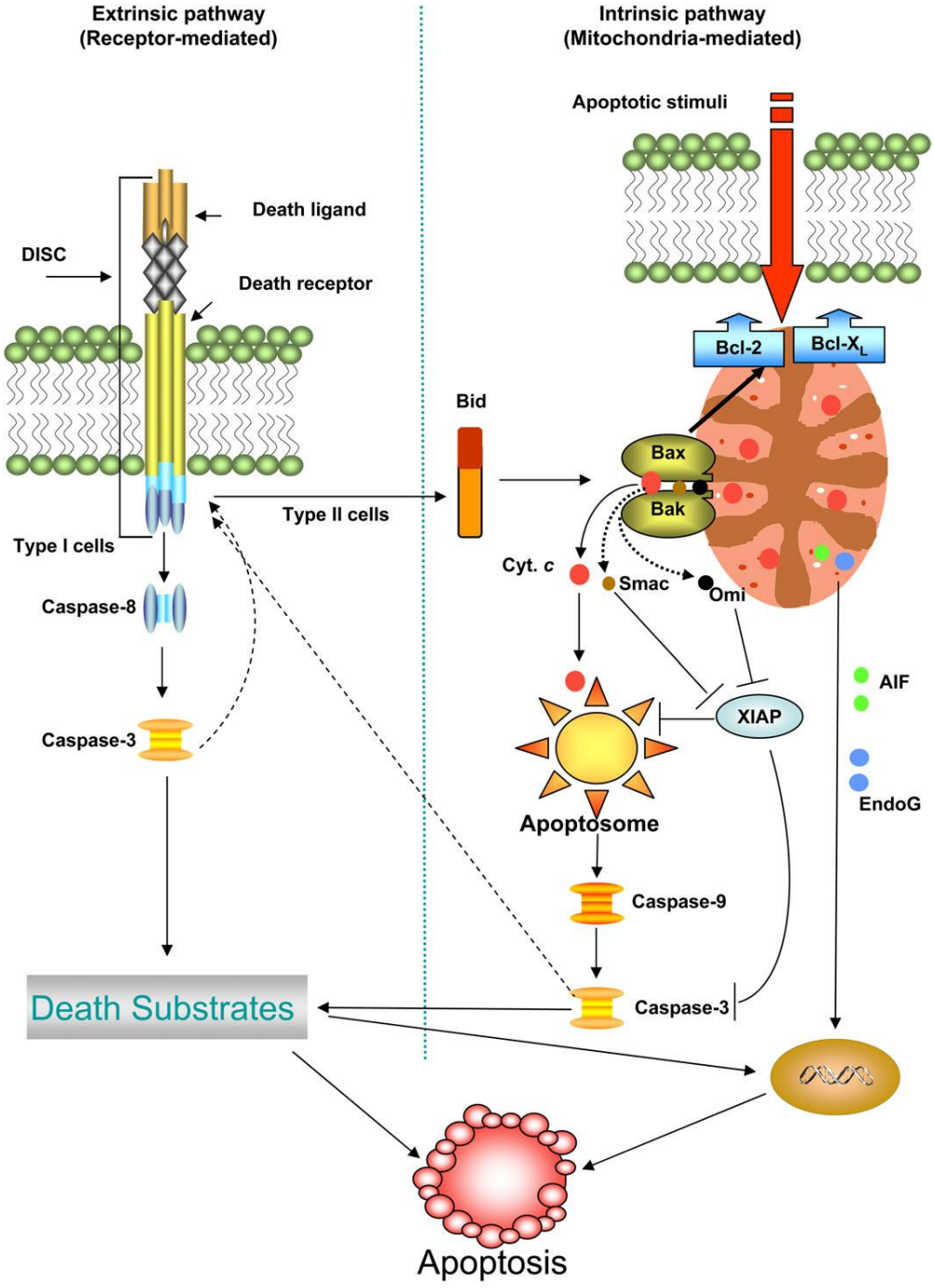

Figure 9. The extrinsic and intrinsic apoptotic pathways. The *extrinsic pathway* is triggered by ligands binding to receptors on the cell surface, resulting in their oligomerization, formation of death-inducing signaling complex (DISC), and caspase-8 activation. In type I cells, caspase-8 activates caspase-3, which results in the cleavage of a host of target proteins and apoptosis. In type II cells, caspase-8 cleaves Bid, resulting in the engagement of the mitochondrial pathway. The *intrinsic pathway* involves the release of proapoptotic proteins (cytochrome c, Smac, Omi, AIF, and EndoG) from the mitochondrial intermembrane space into the cytosol *via* Bax/Bak-mediated pores in the outer membrane. Here, cytochrome c, Smac and Omi participate in the activation of the caspase cascade, whereas AIF and EndoG are further translocated to the nucleus, where they participate in chromatin condensation and large-scale DNA fragmentation. X-Inhibitor of Apoptosis Protein (XIAP) is a cytosolic IAP, whose activity is blocked by Smac and Omi. Bcl-2 and Bcl-X_L inhibit apoptosis. (Orrenius et al 2011)

b) Necrosis

In contrast to apoptosis and autophagy, the swelling of organelles and rupture of the plasma membrane during necrosis result in the release of many inflammatory molecules, leading to an inflammatory respons. Necrosis is prominent in ischemia, trauma and some forms of neurodegeneration (Golstein and Kroemer 2007, Vanlangenakker *et al.* 2008). For a long time, necrosis has been considered merely as an accidental uncontrolled form of cell death, but evidence is accumulating that the execution of necrotic cell death may be finely regulated by a set of signal transduction pathways and catabolic mechanisms (Kroemer *et al.* 2009). Some authors therefore have proposed the term 'necroptosis' to indicate regulated – as opposed to accidental – necrosis (Vanden Berghe *et al.* 2010). Necroptosis is biochemically defined as a form of cell death that is activated by the receptor-interacting protein kinase 1 (RIPK1) and RIPK3, a metabolic burst associated with overproduction of reactive oxygen species (ROS), as well as mitochondrial and lysosomal membrane permeabilization, ATP depletion, failure of Ca^{2+} homeostasis and perinuclear clustering of organelles (Golstein and Kroemer 2007, Vanlangenakker *et al.* 2008, Vandenabeele *et al.* 2010).

c) Autophagy

In contrast, autophagy is not associated with phagocytosis, and is characterized by the presence of autophagic vacuoles that fuse with lysosomes to form autophagolysosomes within the dying cells that are responsible for self-degradation (Orrenius *et al.* 2011). It is an evolutionary conserved mechanism that, in addition to its role in the normal turnover of proteins and organelles, is involved in the cell's response to stress and starvation (Gump and Thorburn 2011). Although the classification into different modes of cell death is useful, there is considerable overlap between the different mechanisms, particularly *in vivo* (Gump and Thorburn 2011, Orrenius *et al.* 2011). There is accumulating evidence that the inhibition of autophagy is oncogenic. Loss of only one allele from either one of the two autophagy genes, beclin-1 or UVRAG (UV radiation resistance-associated gene), is sufficient to promote carcinogenesis. For example, mutation in beclin-1 is common in human cancer (Koneri *et al.* 2007). Multiple oncogenes, such as Bcl-2 and Akt, inhibit autophagy, supporting the hypothesis that autophagy contributes to tumor suppression (Sridharan 2011). Active autophagy can prevent tumor development by at least two mechanisms. It may switch the

balance between protein synthesis and degradation towards degradation to reduce cell growth. Alternatively, it may act to remove defective mitochondria and other intracellular organelles that are potential sources of ROS, which can induce DNA damage and genome instability and thereby promote carcinogenesis (Orrenius *et al.* 2011).

4.2. Gap junctions and cell death

A body of evidence shows that GJIC is associated with apoptotic activity. Communication *via* GJs frequently underlies the propagation of cell death between a dying cell and its healthy neighbor; this phenomenon of 'bystander death' ('the kiss of death') has gained a lot of attention in the context of ischemia-related cell injury, such as in the case of cerebral infarction (Lin *et al.* 1998, Contreras *et al.* 2004, Nakase *et al.* 2004) and to amplify the potency of cancer treatment (Krutovskikh *et al.* 2002). Recently, Feine *et al.* showed that a localized oxidative insult of endothelial cells propagates through GJIC while stimulating de-novo generation of reactive oxygen and nitrogen species, thereby driving the insult's expansion (Feine *et al.* 2012). Previous work from our group as well as others demonstrated that CytC-induced apoptosis propagates from dying to healthy neighboring cells through GJs (Cusato *et al.* 2003, Frank *et al.* 2005, Udawatte and Ripps 2005, Cusato *et al.* 2006, Decrock *et al.* 2009, Peixoto *et al.* 2009). The modulation of IP_3 receptors by Bcl-2 family proteins (Distelhorst and Bootman 2011) will determine the Ca^{2+} load of mitochondria and will thus be expected to be decisive in initiating apoptosis through mitochondrial CytC release. The biochemical nature of the signals that propagate the cell death message are largely unknown. Ca^{2+} has been proposed as the 'killing messenger', thus spreading the death wave from cell to cell. The onset of apoptosis is indeed frequently accompanied by drastic alterations in cytoplasmic Ca^{2+} concentrations, and several crucial apoptotic effectors are known to depend on Ca^{2+} (Krutovskikh *et al.* 2002, Contreras *et al.* 2004, Krysko *et al.* 2005, Cusato *et al.* 2006, Decrock *et al.* 2009). However, Ca^{2+} may be limited by its interactions with more slowly mobile Ca^{2+}-binding proteins. IP_3, produced by PLC activation and triggering Ca^{2+} release from the ER, is probably a better candidate as it can pass through GJs (Boitano *et al.* 1992, Decrock *et al.* 2012). Its ER IP_3-receptor is modulated by pro- and anti-apoptotic proteins such as Bcl-2 (Chen *et al.* 2004, Rong *et al.* 2008), and the Ca^{2+} changes may trigger CytC release from mitochondria (Orrenius *et al.* 2003). Recently Decrock *et al.*

stated that the transfer of IP_3 through GJs is critical, but was not sufficient for the spread of apoptosis (Decrock *et al.* 2012). Other proposed candidate killer messengers that can pass through GJs include cAMP and cGMP (Seul *et al.* 2004, Krysko *et al.* 2005). Potential rescue messengers on the other hand include glucose and ATP or the free radical scavenger ascorbic acid and reduced glutathione that are all able to pass through GJs and favor cell survival (Contreras *et al.* 2004). Likewise, GJs can promote cell survival by limiting the flux of toxic metabolites, such as nitric oxide and superoxide ions (Nakase *et al.* 2003, Contreras *et al.* 2004, Nakase and Naus 2004). Thus, there seems to be a clear two-way traffic between (early) apoptotic and non-apoptotic cells, and depending on the balance, two opposite biological outcomes can be achieved, namely cell death or cell survival (Vinken *et al.* 2006).

4.3. Connexin hemichannels and cell death

As mentioned in *section 1.4. Connexin hemichannels*, CxHCs may open under conditions often regarded as stressful to cells and HC opening may lead to cell death, caused by the excessive entry of Na^+ and Ca^{2+} ions and the loss of extracellular ATP or other crucial molecules (Kalvelyte *et al.* 2003, Saez *et al.* 2003, Contreras *et al.* 2004). Recent evidence from Decrock *et al.* showed that Cx43 HCs contribute to the propagation of apoptotic cell death in a rat C6 glioma cell model (Decrock *et al.* 2009). In ischemia-related cell injury, the involvement of HCs as 'toxic pores', has been demonstrated. Indeed, metabolic inhibition of astrocytes was found to accelerate cell death, which was directly associated with Cx43 HC opening (Contreras *et al.* 2004, Retamal *et al.* 2007). The releasing molecules themselves (e.g. ATP, glutamate or prostaglandins), can also enroll as paracrine communication components and ligand-receptor binding of these messengers is often accompanied by a rise in cytosolic Ca^{2+} (and trigger a new cycle of HC responses)(Orrenius *et al.* 2003). Increased cytosolic Ca^{2+} concentration may exert toxic effects on neighboring cells. On the other hand, HCs might favor cell survival. A well-known example includes their involvement in the anti-apoptotic actions of bisphosphonates. Alendronate inhibits apoptosis in osteoblasts and osteocytes, by a mechanism that involves the activation of ERK/MAP kinases and Src kinase. It is thought that alendronate induces opening of the Cx43-based HCs. The subsequent conformational change probably causes activation of the Cx43-associated Src kinase. The latter then triggers ERK/MAP kinases, which in turn results in cell survival and thereby the

prevention of osteoporosis (Goodenough and Paul 2003, Plotkin *et al.* 2005). Another possible link between HCs and cell survival/death relates to modifications in the subcellular localization of HCs. For example, upon ischemic preconditioning which is a form of cardioprotection, Cx43 translocates from the plasma membrane to the inner mitochondrial membrane through a heat-shock protein 90-dependent pathway (Boengler *et al.* 2005, Rodriguez-Sinovas *et al.* 2006). Cx43 redistribution to mitochondria was also shown in endothelial cells and could have a role in endothelial dysfunction. Li *et al.* showed that homocysteine-exposed human umbilical vein endothelial cells (HUVEC) overexpress Cx43 and homocysteine induced a Cx43 shift from intercellular plaques to intracellular compartments (Li *et al.* 2002). Homocysteine decreased EDHF-mediated relaxations in rat interlobar arteries, suggesting that Cx43 redistribution to mitochondria could have a role in endothelial dysfunction (Li *et al.* 2002). Mitochondrial Cx43 was also found to be a major regulator of cardiomyocyte apoptosis (Goubaeva *et al.* 2007).

4.4. Connexin proteins and cell death

Connexin proteins as such can influence tissue homeostasis by actions that are not related to their channel-forming capabilities. Connexin proteins can directly or indirectly affect the production of cell growth and cell death regulators or can act as a cellular signaling reservoir by reversibly interacting with these substances (Vinken *et al.* 2012). It has been suggested that connexins participate in cell death pathways through direct interaction with apoptotic factors. The cytoplasmic co-localization of Cx26 and Cx43 with the Bcl-2 proteins Bak, Bcl-X_L and Bax, in human breast cancer cells and human colorectal cancer cells, respectively, could be in favor of this idea (Kanczuga-Koda *et al.* 2005, Kanczuga-Koda *et al.* 2005). Connexins may also be involved in the control of cell death-related gene expression. A number of papers have reported changes in the expression of individual subsets of apoptotic factors while interfering with connexin expression. For example, in heart tissue of Cx43 knockout mice, an altered expression of the apoptotic genes Bok, Bax, Bid, Diablo, caspase 6 and caspase 9 was shown (Walker *et al.* 2005). The exact nature of the link between connexins and apoptosis-related gene expression is still a matter of debate. One hypothesis is that connexins are directly involved in transcriptional control and modulate gene expression for instance in rat osteocytes (Stains and Civitelli 2005). Connexin proteins can also interact with

cell growth and cell death regulators, such as the adherens junction protein β-catenin and the tight junction building blocks ZO-1/2 and ZO-1 associated nucleic acid binding protein (Jiang and Gu 2005).

Figure 10 summarizes the role of connexins/pannexins and their channels in cell death signaling.

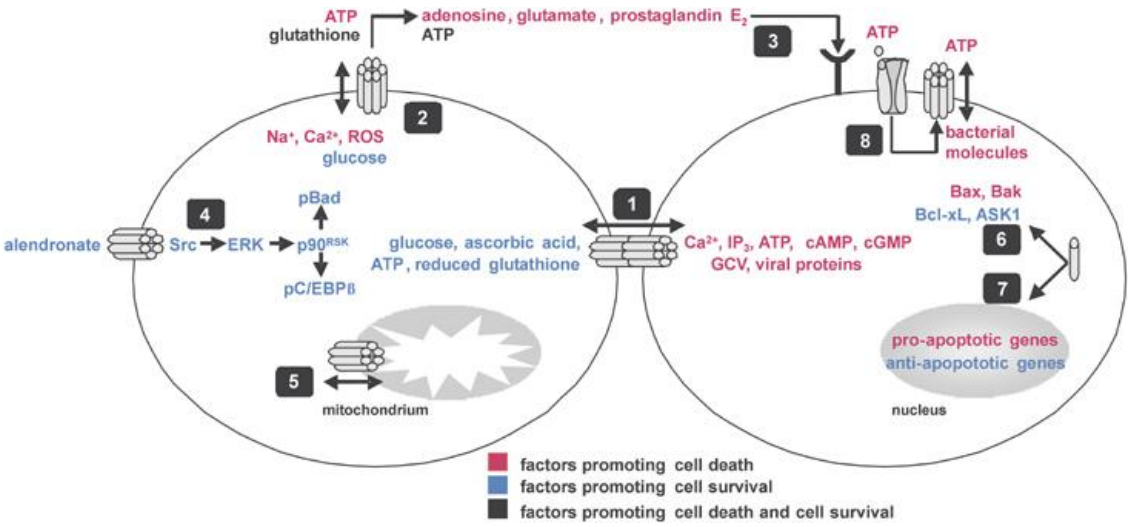

Figure 10. Overview of connexin- and pannexin-related signaling in cell death. Connexins can affect the cell death process through GJs (1), HCs (2) or connexin proteins (6,7). GJ channels can support direct exchange of cell death and cell survival signals between cells (1). HCs may contribute to cell death by four different mechanisms: by the entry of cell death or the loss of cell survival signals (2), through paracrine signaling of death or survival messengers (3) by HC-mediated transmembrane signal transduction (4) or by affecting mitochondrial functioning (5). Cx proteins as such can associate with cell death regulators (6) or influence the expression of these molecules (7). HCs composed of pannexins may act as a permeabilization pore by itself or as a part of the P_2X_7R death complex (8), allowing ATP to leave the cell or bacterial molecules to make their way into the cell. (ASK1, apoptosis signal-regulating kinase 1; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ERK, extracellular signal-regulated kinase; GCV, ganciclovir; IP_3, inositol trisphosphate; pBad, phosphorylated Bad; pC/EBPß, phosphorylated CCAAT/enhancer-binding protein ß; ROS, reactive oxygen species) (Decrock *et al.* 2009)

Chapter III: Vascular grafts

1. Introducing vascular grafts

Vascular grafts have been widely used for myocardial reperfusion following coronary artery occlusion or for major peripheral vascular diseases. The superiority of autogenous veins in reconstructive procedures is well recognized (Rashid *et al.* 2004). This is primarily due to the fact that the risk of rejection associated with antigen-antibody mismatch may be eliminated. Also the process of harvesting and transplanting the vessel is almost instantaneous, reducing the concentration of damage associated with storage of tissue in cold ischemia. However, an inadequate supply of these vessels in patients has resulted in surgeons having to use alternative techniques (Thakrar *et al.* 2006). Prosthetic vessels are an option but they are insufficient to match the functional properties of autogenous vessels, especially relating to the biomechanical properties (Hoenig *et al.* 2005). Expanded polytetrafluoroethylene (ePTFE) is one of the most frequently used prostheses in human clinical practice. This biomaterial had several advantages over other prosthetic materials, including high electronegativity and a hydrophobic surface that reduces platelet adhesion. Although this material boasts a good blood/biomaterial interface and shows excellent biotolerance on the part of the recipient, many polymeric materials are to some extent thrombogenic. Attempts to resolve this problem include establishing a cell lining on the luminal surface of the graft using autologous endothelial cells from the patient. Coating the luminal surface in this way substantially improves clinical results after grafting, increasing the biocompatibility, and viability of the prostheses (Deutsch *et al.* 1997, Laube *et al.* 2000). However, prosthetic material is also susceptible to infection, a devastating complication that often requires its withdrawal and replacement (Pascual *et al.* 2004). To reduce such risks, it has been proposed to replace the infected graft by human vessels collected from clinically dead patients. This alternative source of blood vessels, named allografts, has the advantage that they are relatively more abundant than autografts and more likely to possess adequate biomechanical properties to function efficiently in the recipient. Conversely, there are common problems associated with the use of this technique, primarily being an increased risk of disease transmission between individuals. Further, rejection is a common occurrence in patients and occlusion of a graft is the most serious postoperative complication. Many factors have been considered responsible for the early occlusion of grafts. Atherogenic factors such as smoking, diabetes, dyslipidaemias and high blood pressure cause significant

changes in the vascular wall that lead to a reduction in viability when those vessels are used as grafts (Kanellaki-Kyparissi *et al.* 2005). Vein and artery bypass grafting is an integral component of cardiovascular surgical practice for both arterial and venous diseases. However, many of these grafts will eventually fail due to either intrinsic or extrinsic causes such as intimal hyperplasia, accelerated atherosclerosis, thromboembolism and graft sepsis (Davies and Hagen 2011). Veins are the most commonly used source of vessel and the general consensus is that arteries are more antigenic (Elmore *et al.* 1991, Fujitani *et al.* 1992). Venous grafts (saphenous veins) are mostly used for femorodistal bypasses on crural or pedal vessels in the absence of a suitable autologous saphenous vein. These grafts are resistant to infection when performed for revascularization in patients with an infected ulcer (Randon *et al.* 2010). Arterial grafts (femoral arteries) are suited for aorta-iliacal bypasses after infection. Infection is the principal indication for using an arterial allograft and this may be a primary arterial infection in which haematogenous spread of bacteria or fungi cause an infective arteritis or due to trauma or iatrogenic injury such as during angiographic procedures; or a secondary infection to previous arterial reconstructive surgery (Dodd 2010). Although long term follow-up of bypass patients showed that the patency of arterial grafts was greater than that of venous grafts and for that reason the use of the internal thoracic artery has been steadily gaining ground in recent years (Kanellaki-Kyparissi *et al.* 2005).

2. Preservation of vascular grafts

To date, three current approaches to vessel preservation exist. The first is storage of the vessels at hypothermic temperatures (4 °C, for approximate 1 month). A second preservation and the most used technique is cryopreservation, a process of long-term subzero preservation of blood vessels. Here, vessel segments are placed in 10-15 % cryoprotectants and cooled slowly in a freezer to –70 °C (yielding an average cooling rate of –1-2 °C/min). Then the vessels are placed in liquid nitrogen, cooled to –196 °C (Pegg 2002, Thakrar *et al.* 2006). Another subzero technique is vitrification; this process involves converting an aqueous solution by use of high concentrations of cryoprotectants into an amorphous solid at low temperatures, such that when the liquid is cooled down it may be taken below its melting point without producing ice crystal growth. Restriction of ice crystal growth results in reduced structural and molecular damage. However, the key problem associated with vitrification of tissues is that approximately half the water within the tissue must be replaced by the vitrification solute. The vessels are further rapidly cooled to –196 °C by placing the tube in liquid nitrogen (with an average cooling rate of –40 °C/min) (Thakrar *et al.* 2006). Fresh vascular grafts undergo rapid rejection and destruction. Preserved grafts were thought to be less so and arterial banks became standard in many vascular centers. Harvesting, sterilizing, and preserving grafts utilized formalin, alchohol, glycerine, ethylene oxide, betapriolactone, high voltage cathode ray irradiation, and freeze drying, each had its shortcomings (Callow 1996, Dodd 2010). However, recently long term-storage at 4 °C in tissue culture medium of vein homografts was successful for distal revascularization in critical ischemia, especially in infected fields (Galambos *et al.* 2009).

Over the last years, cryopreserved vessels are being increasingly employed in vascular reconstruction procedures, given the developments made in minimizing tissue damage induced by low temperatures. It has been demonstrated that storage at -196 °C for an extended period of time yields a better preservation of vascular function than storage at -70 °C (Rigol *et al.* 2000). However, other studies have found that storage of vessels at a temperature of -80 °C seemed to lead to better results than temperatures as low as -160 °C / -190 °C (Ku *et al.* 1994). Storage of allografts in liquid nitrogen up to 13 years did not significantly undergo loss of cell viability in cardiac valve allografts (Mirabet *et al.* 2008).

Further research pointed to an important role for the cooling rate and medium to improve vascular function of cryopreserved vessels. Cryopreservation with a cooling rate of 0.7 °C per minute seemed to be superior above the standard 1 °C per minute cooling in porcine femoral arteries (Rigol *et al.* 2000) and rabbit common carotid arteries (Song *et al.* 1995). In human internal mammary arteries the optimal freezing rate for preserving post-thaw contractile function is around 1 °C/min and for human saphenous veins 0.6 °C/min (Muller-Schweinitzer 2009). An even more important factor in improving the vessel quality after cryopreservation is the thawing procedure. Rapid thawing was associated with more cell damage and fractures (see further) of the grafts. Slow, automated thawing at a warming rate of 1 °C per minute improved vessel quality and endothelial preservation (Pascual *et al.* 2004) especially in arteries (Muller-Schweinitzer 2009). Today, slow automated thawing is highly recommendable for arterial grafts, while venous grafts are less susceptible to rapid thawing (Ruddle *et al.* 2000). Further advances in the cryopreservation/thawing process (Bujan *et al.* 2000, Bujan *et al.* 2001) including the introduction of cryoprotectants (Gournier *et al.* 1995, Alonso *et al.* 1999, Castier *et al.* 1999) have led to their current use in clinical practice. Glycerol was the first cryoprotectant agent to gain widespread use in cryobiology, for cryopreserving red blood cells and sperm. The value of dimethyl sulfoxide (DMSO) as a cryoprotectans was discovered not long thereafter. Other polyols, such as ethylene glycol (automobile anti-freeze) and propylene glycol (formerly used to reduce ice formation in ice cream) were later shown to be effective cryoprotectants (Pegg 2002). In the late seventies, cryopreservation of canine artery segments in liquid nitrogen and 15 % DMSO, an oxygen radical scavenger, was found as an excellent method for preserving and storing fresh allograft arteries (Boren *et al.* 1997). DMSO, the most commonly used cryoprotectant of vascular tissues (Muller-Schweinitzer 2009) rapidly diffuses through cell membranes and prevents the formation of intracellular ice crystals. Optimal protection against cryoinjury will be obtained by the combination of a permeating and non-permeating cryoprotecting agent (CPA). Glycerol and DMSO are permeating CPAs and examples of non-permeating CPAs are sucrose and glucose and are suggested to stabilize the cell volume by retaining more liquid water at low temperatures, thereby reducing the external electrolyte concentration (Muller-Schweinitzer 2009).

Today, the performance of cryopreserved vascular substitutes is not ideal, mainly because of spontaneous fractures (Figure 1) appearing at the time of thawing or grafting (Lehalle *et al.* 1997, Pegg *et al.* 1997, Bujan *et al.* 2000). Cryopreserved vessels suffer from macroscopic and microscopic fractures; a phenomenon that probably results from the thermal stresses created by rapid warming of the vitreous material that is produced by freeze-concentration of the aqueous phase. Relatively slow warming to -100 °C, at which temperature the vitreous material has softened, reduces these stresses and avoids fractures (Pegg *et al.* 1997).

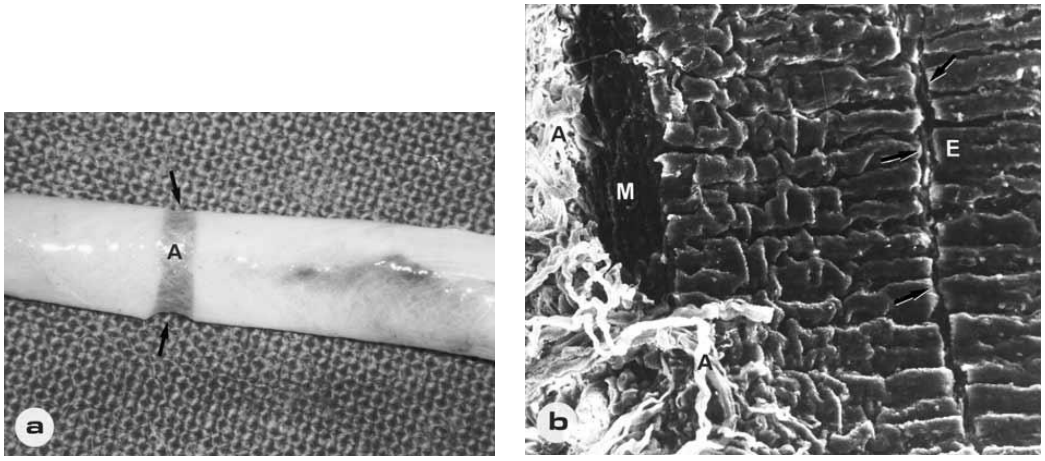

Figure 1. Spontaneous fractures in a blood vessel. a. a cryopreserved/rapidly thawed arterial segment. Note how the spontaneous fracture affects the entire wall (arrows) except the tunica adventitia (A). ×25. b. SEM of the fracture above, showing clean cutting of the muscle layer (M) and disorganized fibres in the adventitia (A). Also, on the luminal surface, a microfracture (arrows) may be seen to only involve the endothelium (E) and the internal elastic lamina. ×200. (Bujan *et al.* 2000)

3. Cell death in cryopreserved vascular grafts

One of the main factors leading to failure of grafting procedures performed using cryopreserved vessel substitutes is, besides spontaneous fractures, the endothelial loss provoked by the methods of vascular cryopreservation used at most human vessel banks (Pascual *et al.* 2004). The loss of the endothelial layer could be the consequence of several factors: the freezing protocol used, the cryopreservation medium, the cold storage temperature or the thawing protocol (Pegg *et al.* 1997). The endothelium is the key regulator of vascular homeostasis. Endothelial cells continuously interact with blood components and with the structures of the vessel itself. Endothelial function is essential for maintaining vessel tone and antithrombotic properties. Hence, its adequate preservation plays a crucial role in the mid- and long-term viability of arterial grafts (Rigol *et al.* 2000). Besides maintaining a non-thrombogenic surface and regulating vessel tone, the main functions of the vascular endothelium are: controlling vascular cell growth (Jurado *et al.* 2002), cytokine regulation, maintaining selective permeability, synthesizing extracellular matrix components, and the synthesis, secretion and metabolism of biologically active components. The partial absence of the endothelium means that the extracellular matrix makes contact with the circulating blood, which generally leads to several acute disease states such as thrombosis and chronic/progressive states such as hyperplasia (Bellon *et al.* 1996) and atherosclerosis. Several factors are responsible for the cellular damage in cryopreserved vascular grafts. Toxicity of the preserving agents is one factor. Therefore a maximum concentration of 15 % DMSO is advised. DMSO as well as other cryoprotecting agents are important to prevent intracellular ice formation. Recently it has been shown that this ice formation is more lethal in cells in suspension than in monolayers. This was due to the presence of GJs in monolayers and the abililty of ice propagation between cells *via* GJs (Armitage and Juss 2003, Zhurova *et al.* 2010). MDCK cells expressing no GJs showed indeed no increased damage to intracellular freezing (Armitage and Juss 2003). In HepG2 cell pairs, the GJ blocker 18β glycyrrhetinic acid reduced the intercellular ice propagation rate compared to untreated controls (Irimia and Karlsson 2002). Thawing procedures are another important factor leading to impaired vessel viability. Rapidly thawed endothelial cells showed irreversible ultrastructural damage such as mitochondrial dilation and rupture, reticular fragmentation and peripheral nuclear condensation. In contrast, slow gradually

thawing showed a lower proportion of damaged cells. Gradual automated thawing seemed to be the best way of preserving the endothelial surface of the arterial cryograft (Pascual *et al.* 2004).

Chapter IV: AIMS

In this doctoral thesis, we investigated the role of cannabinoids and CGRP on K^+ currents in acutely isolated small mesenteric rat artery SMCs and the role of CxHCs in $[Ca^{2+}]_i$ dynamics and contraction in this blood vessel type. While GJs and CxHCs modulate vessel tone, they may also contribute to cell death processes in pathological conditions. We further determined whether inhibition of these connexin channels could protect vascular grafts against cryopreservation-induced cell death.

Influence of methanandamide and CGRP on K^+ currents in smooth muscle cells of small mesenteric arteries

Cannabinoids have potent vasodilatory actions in a variety of vascular preparations. Their mechanism of action, however, is complex. Apart from acting on vascular smooth muscle or endothelial cannabinoid receptors, several studies point to the activation of TRPV1 receptors on primary afferent perivascular nerves, stimulating the release of CGRP. We explored possible direct actions of methanandamide and of CGRP on rat small mesenteric artery SMCs by comparing their effects on whole cell K^+ currents in freshly isolated myocytes. We specifically addressed the question whether CGRP might activate BK_{Ca} currents in these cells.

Connexin hemichannels contribute to Ca^{2+} dynamics and contractility of smooth muscle cells in small mesenteric arteries

GJs are important for the communication between vascular cells and the synchronization of Ca^{2+} signals and control the vessel diameter and blood flow. Non-junctional CxHCs reside in the plasma membrane in a closed state. HCs can be opened by various messengers and conditions, including $[Ca^{2+}]_i$. HC opening may also influence $[Ca^{2+}]_i$ and recent evidence suggests this may play a role in Ca^{2+} oscillations. Previous reports pointed to an important role for GJs in vascular tone, however, unapposed HCs have never been investigated as a putative additional partner in controlling the vessel wall tone. The connexin mimetic peptide Gap27 inhibits Cx37 and Cx43 HCs after short incubation, and GJs with a time delay. The TAT-L2 peptide and TAT-CT9 peptide have specificity for Cx43 and the first inhibits HC responses while the latter prevents closure of the HC at high micromolar $[Ca^{2+}]_i$. Here we determine

whether HCs can play a role in SMC $[Ca^{2+}]_i$ dynamics and contraction. We used acutely isolated small mesenteric blood vessels to this purpose and investigated both $[Ca^{2+}]_i$ dynamics and contractility of the vessels induced by exposure to norepinephrine.

Inhibiting connexin channels protects against cryopreservation-induced cell death in human blood vessels

Cryopreserved vascular grafts are widely used for cardiovascular interventions. However, the graft quality is suboptimal due to the cryopreservation protocol used in vascular banks. Grafts are preserved in cold storage at 4 °C as well as cryopreserved, but the procedure is still unsatisfactory and complication such as thrombosis and vasospasms occur and could lead to late graft failure. CxHCs and GJs play a role in cell – cell communication and cell death processes. GJs can pass messenger molecules and metabolites which can influence neighbouring cells. Under pathological conditions, this pathway could induce cell death of neighbouring cells or pass cell death messengers. HCs are normally closed but may open under stressful conditions and thereby promoting cell death. Uncontrolled opening of HCs can contribute to loss of ATP, entrance of Ca^{2+} and disturbing of ion gradients. All these items form an important factor that could lead to cell death. Blocking GJs and HCs by connexin mimetic peptides could prevent cell death after cryopreservation. An example of a connexin mimetic peptide with possible interesting effects on vascular cells is Gap27. This peptide has a peptide sequence identical to a short amino acid sequence on the second extracellular loop of Cx43 and Cx37. Short incubation of this peptide is sufficient to inhibit HC related responses, while long incubations are necessary to inhibit GJs. We hypothesized that during prelevation and preservation of blood vessels, inhibiting both GJs and HCs might reduce cell death in vascular grafts. We investigated the role of connexin mimetic peptides on cell viability in vascular grafts.

Chapter V: Influence of methanandamide and CGRP on K^+ currents in smooth muscle cells of small mesenteric arteries

Influence of methanandamide and CGRP on K^+ currents in smooth muscle cells of small mesenteric arteries

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Abstract

Cannabinoids have potent vasodilatory actions in a variety of vascular preparations. Their mechanism of action, however, is complex. Apart from acting on vascular smooth muscle or endothelial cannabinoid receptors, several studies point to the activation of type 1 vanilloid (TRPV1) receptors on primary afferent perivascular nerves, stimulating the release of calcitonin gene-related peptide (CGRP). In the present study, the direct influence of the cannabinoid methanandamide and the neuropeptide CGRP on the membrane potassium ion (K^+) currents of rat mesenteric myocytes was explored. Methanandamide (10 μM) decreased outward K^+ currents, an effect similar to that observed in smooth muscle cells from the rat aorta. Conversely, CGRP (10 nM) significantly increased whole cell K^+ currents and this effect was abolished by pre-exposure to TEA (1 mM) or iberiotoxin (100 nM), inhibitors of large-conductance calcium-dependent K (BK_{Ca}) channels but not by glibenclamide (10 μM), an inhibitor of ATP-dependent K (K_{ATP}) channels. In the presence of the CGRP-receptor antagonist $CGRP_{8-37}$ (100 nM), the adenylyl cyclase inhibitor SQ22536 (100 μM), or the PKA inhibitor Rp-cAMPS (10 μM), CGRP had no effect. These findings show that methanandamide does not increase membrane K^+ currents in smooth muscle cells of small mesenteric arteries, supporting an indirect mechanism for the reported hyperpolarizing influence in this vessel. Moreover, CGRP acts directly on these smooth muscle cells by increasing BK_{Ca} channel activity in a CGRP receptor and cyclic adenosine monophosphate (cAMP) dependent way. Collectively, these data indicate that methanandamide relaxes and hyperpolarizes intact mesenteric vessels by releasing CGRP from perivascular nerves.

Keywords: cannabinoids, vasoactive agents, endothelial factors, vasorelaxation, vanilloid receptor, neuropeptides.

INTRODUCTION

Anandamide has been identified as an endogenous counterpart of Δ9-tetrahydrocannabinol, the psychoactive component of marijuana. The endocannabinoid is synthesized by cleavage of the lipid precursor arachidonyl phosphatidylethanolamine, and is metabolized relatively quickly to arachidonic acid and ethanolamine. The potent vasodilatory influence of anandamide has been shown in a variety of isolated vascular preparations. Its mechanism of action, however, is complex and seems to vary with species, vessel type, and even vessel size (Vanheel and Van de Voorde 2000, O'Sullivan *et al.* 2004). In rabbit pial arterioles and mesenteric arteries, the anandamide induced vasodilation is mediated by the generation of vasodilator eicosanoids, since it is blocked by indomethacin and diclofenac (Ellis and Conanan 1995, Fleming *et al.* 1999). Similarly, in isolated bovine coronary arteries the vasorelaxant effect of anandamide has been suggested to be due to its hydrolysis to arachidonic acid and further conversion to vasodilatory eicosanoids (Pratt *et al.* 1998). In rat mesenteric arteries, however, the vasorelaxing influence of anandamide is unaffected by cyclooxygenase inhibition (White and Hiley 1998). Moreover, also the stable analogue of anandamide, methanadamide, is a powerfull vasodilator of these vessels (Ralevic *et al.* 2000).

Endogenous, plant derived and synthetic cannabinoids have been reported to target receptors different from the classical CB1 and CB2 receptors. In the rat mesenteric and coronary circulation, a novel non CB1/non CB2 receptor present on endothelial cells, has been shown to be involved in vasorelaxation (O'Sullivan *et al.* 2004). Furthermore, an action on other receptors and membrane proteins was described. Cannabinoids have been shown to activate endothelial TRPV4 receptors (Watanabe *et al.* 2003) and endothelial peroxisome proliferator-activated receptor gamma (O'Sullivan *et al.* 2005), and anandamide has been demonstrated to bind to serotonin (5-HT)-3 receptors (Fan 1995) and modulate the activity of the NMDA receptor (Hampson *et al.* 1998). Furthermore, stimulation of TRPV1 receptors on perivascular sensory nerves accounts for the vasorelaxing influence of anandamide (Zygmunt *et al.* 1999). Other studies showed that cannabinoids directly inhibit calcium channels (Gebremedhin *et al.* 1999, Breyne *et al.* 2006) and activate various K^+ channels (Randall and Kendall 1997, White *et al.* 2001, Sade *et al.* 2006) an influence which might also account for its vasorelaxant action. By contrast, cannabinoids have direct inhibitory effects

on delayed rectifier K^+ currents in freshly isolated rat aortic smooth muscle cells (Van den Bossche and Vanheel 2000).

Calcitonin gene related peptide (CGRP) is a 37 amino acid peptide that is generated by alternative splicing of the calcitonin gene transcripts. As a neurotransmitter it is localized predominantly in sensory nerves, innervating various tissues and blood vessels. In rat hepatic and small mesenteric arteries, the vasorelaxant influence of anandamide was antagonized by the TRPV1 receptor antagonist capsazepine and the CGRP receptor antagonist CGRP(8-37) but not by the classical CB1 receptor antagonist SR141716A (Zygmunt *et al.* 1999). It was proposed, therefore, that cannabinoids exert their relaxing influence by stimulating the release of CGRP from perivascular nerves (Zygmunt *et al.* 1999).

The mechanism involved in vasorelaxation to CGRP seems to vary among species and vessel types. In the rat aorta (Gray and Marshall 1992) and pulmonary arteries (Wisskirchen *et al.* 1998), human omental and bovine retinal arteries (Boussery *et al.* 2005), CGRP interacts with endothelial receptors stimulating the release of endothelium derived relaxing factors, as both endothelium removal and blocking NO synthesis significantly reduced vasorelaxation. By contrast, in cat cerebral, pig coronary and rat mesenteric vessels, CGRP acts directly on the smooth muscle cells. A number of studies have shown that CGRP relaxes tension by increasing the cyclic AMP content in vascular smooth muscle (Ishikawa *et al.* 1993, Kageyama *et al.* 1993, Yoshimoto *et al.* 1998). The resultant activation of protein kinase A (PKA) and PKA dependent phosphorylation has been shown to open ATP-dependent K^+ (K_{ATP}) channels (Nelson *et al.* 1990, Quayle *et al.* 1994) hyperpolarizing and relaxing the tissue (Wellman *et al.* 1998). In line with this view, we have shown that the hyperpolarization of isolated rat gastric and small mesenteric arteries evoked by methanadamide was abolished by capsazepine (Breyne and Vanheel 2006). Exogenous CGRP produced a hyperpolarization of the smooth muscle cells of comparable time course and magnitude, and both methanandamide- and CGRP-induced membrane potential changes were sensitive to the K_{ATP} channel inhibitor glibenclamide. However, two studies on isolated rat and porcine coronary arteries have failed to demonstrate K_{ATP} channel opening in CGRP-induced vasorelaxation (Prieto *et al.* 1991, Kageyama *et al.* 1993). CGRP might also mediate relaxation by activation of BK_{Ca} channels, by inhibition of voltage gated calcium channels (Breyne *et al.* 2006) or by decreasing the Ca^{2+} sensitivity of the contractile apparatus

(Sheykhzade and Nyborg 2001). In rat gastric arteries, we found the vasorelaxation to CGRP to be partly sensitive to low concentrations of TEA, suggesting involvement of BK_{Ca} channel activation (Breyne *et al.* 2006). In a number of vascular preparations, PKA dependent vasodilators have been shown to activate smooth muscle BK_{Ca} channels (Schubert and Nelson 2001).

In the present experiments, we explored possible direct actions of methanandamide and of CGRP on rat small mesenteric artery smooth muscle cells by comparing their effects on whole cell K^+ currents in freshly isolated myocytes. We specifically addressed the question whether CGRP might activate BK_{Ca} currents in these cells.

MATERIALS AND METHODS

Cell Isolation

Smooth muscle cells from rat small mesenteric arteries were dissociated on the day of the experiments. The animals were treated in accordance with the Guiding Principles for the Care and Use of Animals (published by National Academy Press, 2101 Constitution Ave, NW, Washington, DC 20055, USA, 1996) and the experiments were approved by the ethical committee on animal research of Ghent University. The animals were anesthetized by a lethal dose (200 mg kg^{-1}) of pentobarbitone and killed by cervical dislocation. Third order branches of the superior mesenteric artery from 2-3-month-old female Wistar rats were excised and placed in a chilled solution containing (mM): NaCl, 135; KCl, 5; $NaHCO_3$, 20; $CaCl_2$, 2.5; $MgSO_4.7H_2O$, 1.3; KH_2PO_4, 1.2; EDTA, 0.026; glucose, 10, which was continuously bubbled with 95% O_2 / 5% CO_2. The vessels were carefully cleared of adherent fat and connective tissue, and cut into small pieces. The artery segments were subsequently transferred to cold (4 °C) low calcium dissociation medium (DM) containing (mM): NaCl, 110; KCl, 5; $CaCl_2$, 0.16; $MgCl_2$, 2; KH_2PO_4, 0.5; EDTA, 0.49; NaH_2PO_4, 0.5; Taurine, 10; HEPES, 10; glucose, 10, titrated to pH 7.0. The small pieces were allowed to rest in this medium for 30 min. The tissue was then transferred to fresh DM supplemented with papain (17 U/ml) and bovine serum albumin (2 mg/ml) for 20 min, after which dithiothreitol was added from a concentrated stock solution to a final concentration of 6.7 mM and the mixture was warmed

to 37 °C for 20 min. The partially digested tissue was subsequently washed twice in cold DM. After this, collagenase (410 U/ml) and hyaluronidase (330 U/ml) were added and the mixture was warmed to 37 °C for 20 min. The digested tissue was washed three times in cold DM. After this, gentle trituration with a wide-bore Pasteur pipette yielded a suspension of single cells, which was kept at 4 °C in the low- Ca^{2+} medium.

Electrophysiological recordings

Electrophysiological recordings were performed as previously described (Van den Bossche and Vanheel 2000). Briefly, part of the cell suspension was pipetted into a perfusion chamber fixed to the stage of an inverted microscope (Nikon, Diaphot). After 10 min, allowing the cells to adhere to the glass bottom of the chamber, the cells were superfused with a normal calcium containing solution containing (mM): NaCl, 140; KCl, 5.4; $CaCl_2$, 1.8; MgCl, 1; HEPES, 10; glucose, 10, pH = 7.4.

Whole-cell membrane currents were recorded at room temperature using the conventional patch-clamp technique. Borosilicate glass capillaries were used. Pipettes were attached to the headstage of an EPC-9 patch clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). Pipette resistance ranged 3-5 MΩ after fire-polishing. The composition of the pipette solution for conventional whole-cell recording was (in mM): KCl, 102; K^+ (from KOH), 38; EGTA, 10; NaCl, 10; $CaCl_2$, 1; HEPES, 10; Na_2GTP, 0.2; NaADP, 0.1; Na_2ATP, 3; $MgCl_2$, 3 (pH 7.2, free Ca^{2+} = 17.1 nM). Pipettes were coated with beeswax. After breaking in, the cell membrane capacitance was measured from the capacitive transient using the automatic compensation procedures of the EPC-9 amplifier. Series resistance was compensated by 2-30%. Membrane currents were evoked by stepping the membrane potential from a holding potential of -70 mV for 200 ms to various test potentials between -110 and +60 mV, in 10 mV increments at 3 sec intervals. Currents were filtered at 2.3 kHz and followed on computer monitor. Membrane currents were sampled at 5 kHz, recorded on hard disk and analysed using the Review program of the EPC-9 system. Current values given in the results represent the steady state current, measured as the average current during the terminal 50 ms portion of the voltage step.

In some experiments, measurements of the myocyte membrane potential were performed with the whole cell patch configuration in current clamp mode.

Drugs

Papain, BSA, dithiothreitol, collagenase (type VIII), hyaluronidase (type I-S), glibenclamide, iberiotoxin, tetraethyl ammonium chloride (TEA), 4-aminopyridine (4-AP), SQ22536, and adenosine 5'-triphosphate disodium salt (ATP) were obtained from Sigma Aldrich (St. Louis, MO). Calcitonin gene-related peptide (CGRP rat), $CGRP_{8-37}$, Rp-cAMPS and R-(+)-methanandamide were obtained from Tocris (Bristol, UK).

The influence of a drug was investigated by adding the appropriate amount from a stock solution to a reservoir containing equilibrated superfusion solution a few seconds before use. Pre-exposure times to the various blockers were at least 10 minutes. During superfusion, membrane potential was stepped to 60 mV at 20 seconds interval, in order to observe the influence on the corresponding current and to verify that a steady state effect was reached before recordings of the current family were started. The influences of methanandamide and CGRP reached steady state 4 to 7 minutes after adding these substances to the superfusate.

Stock solutions of methanandamide were made in anhydrous ethanol, while glibenclamide was dissolved in dimethyl sulfoxide. The final concentration of solvent never exceeded 0.1%. CGRP, iberiotoxin, TEA-Cl, 4-AP and Rp-cAMPS were dissolved in water. All concentrations are expressed as final molar concentrations in the superfusate.

Statistics

Results are presented as normalized (i.e. pA/pF) means $\pm$ s.e.mean. Statistically significant differences are evaluated using Student's t-test for paired or unpaired observations, as appropriate. A P value < 0.05 indicates a significant difference. *n* represents the number of cells.

RESULTS

Freshly isolated smooth muscle cells from small mesenteric arteries were superfused at least 10 minutes with the control solution before measurements were started.

1. INFLUENCE OF METHANANDAMIDE AND CGRP ON MEMBRANE CURRENTS

Cells were clamped at a holding potential of -70 mV. Depolarizing voltage steps of increasing amplitude resulted in the activation of a family of outward currents which displayed graded voltage-dependent activation kinetics and showed little inactivation during the 200 ms test pulse (Figure. 1 A).

Influence of methanandamide

The addition of methanandamide (10 µM) to the bath fluid decreased outward K^+ currents of the smooth muscle cells (Figure 1A). The current block by methanandamide reached a steady state approximately 5 minutes after its addition to the superfusate. As we have described for smooth muscle cells from rat aorta (Van den Bossche and Vanheel 2000), the inhibitory influence of the cannabinoid was most pronounced on current inactivation during the 200 ms depolarizing step (Figure 1A). In four experiments, the mean current amplitude measured at the end of the voltage step to +60 mV decreased from 19.3 $\pm$2.6 pA/pF in control conditions to 10.5 $\pm$0.5 pA/pF in the presence of methanandamide. The I-V plot (Figure 1B) revealed a statistically significant inhibition ($P<0.05$) in the potential range of -20 to +60 mV. The methanandamide sensitive (difference) current reversed at -72 mV (Figure 1B inset), indicative for inhibition of a K^+ current.

In the continuous presence of iberiotoxin (100 nM), a selective BK_{Ca} channel inhibitor, inhibition of K^+ currents by methanandamide was still present (Figure 2A). Moreover, after pre-exposure to both 1 mM TEA, at this concentration acting as an inhibitor of BK_{Ca} channels (Langton *et al.* 1991), combined with the K_{ATP} channel inhibitor glibenclamide (10 µM), methanandamide still significantly decreased K^+ currents (Figure 2B). Conversely, after pre-exposure to 4-AP (5 mM), the influence of methanandamide was totally abolished (Figure

2C). These experiments, therefore, point to an inhibition of delayed rectifier K^+ (K_v) currents by methanandamide. In rat aortic smooth muscle cells, indeed, anandamide and methanandamide have been shown to directly affect K_v channels (Van den Bossche and Vanheel 2000).

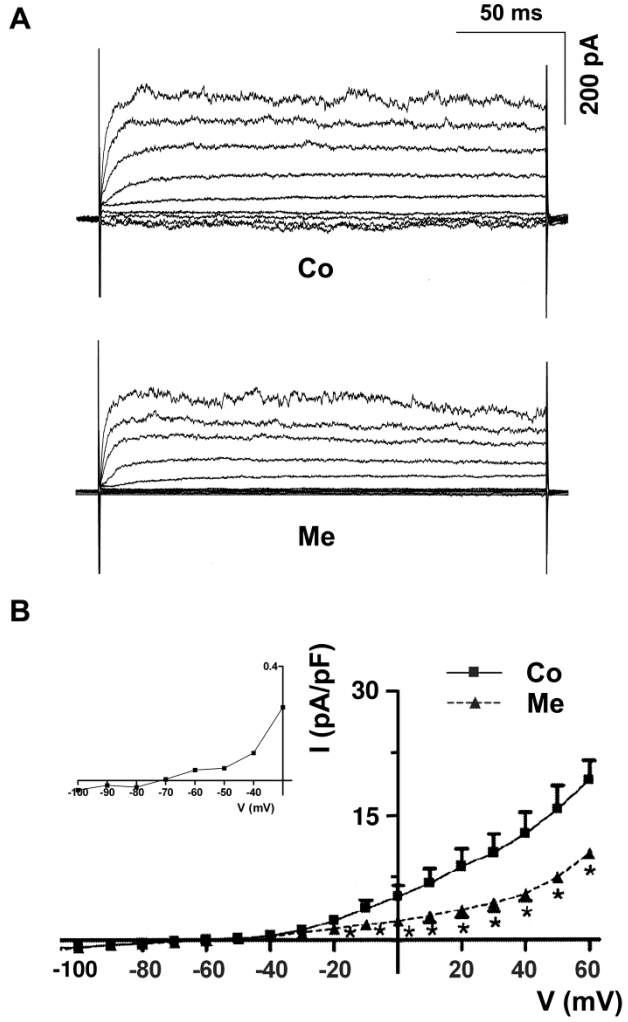

Figure 1. Influence of methanandamide on I_K. A. Representative current traces from a small mesenteric artery myocyte in control conditions (Co) and in the presence of methanandamide (Me, 10 µM). B. Mean normalized current (I)- voltage (V) plot showing the influence of methanandamide on the steady state current. Each point represents the mean + s.e.mean from four cells. * denotes statistically significant from control value at $p < 0.05$. Inset shows the difference current (Co-Me) on an extended current axis.

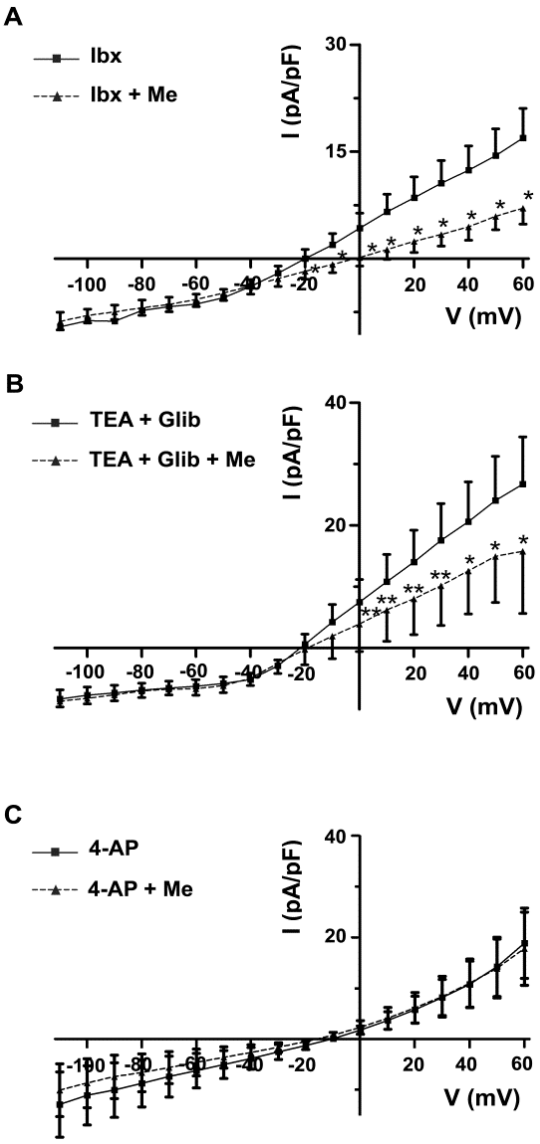

Figure 2. Influence of methanandamide (Me, 10 µM) after blocking BK_{Ca}, K_{ATP} and K_v. A. Mean normalized current (I)-V plot showing the influence of the cannabinoid after pre-exposing the myocytes to iberiotoxin (Ibx, 100 nM). B. Mean normalized IV-plot showing the influence of methanandamide in the combined presence of TEA (1 mM) and glibenclamide (10 µM). Mean normalized IV-plot showing the influence of methanandamide after pre-exposure to 4-AP (5 mM). Each point represents the mean + s.e.mean from 7 (A) or 5 (B, C) cells. * and ** denote statistically significant from control value at $p < 0.05$ or $p < 0.01$, respectively.

Influence of CGRP

Application of the neuropeptide CGRP (10 nM) significantly increased whole cell currents in mesenteric artery smooth muscle cells. Typical traces, obtained after reaching a steady state (which lasted approximately 6 minutes after adding CGRP to the superfusate) are shown in Figure 3A. In eight cells, the mean current amplitude measured at the end of the voltage step to +60 mV was increased to 220% of control levels (Figure 3B). The I-V plot showed a statistically significant increase ($P<0.05$) in the potential range from +40 to +60 mV. The CGRP-induced (difference) current appeared on depolarization beyond +10 mV and showed

pronounced outward rectification. These features suggest the involvement of a voltage dependent potassium channel such as BK_{Ca} (Nelson *et al.* 1990).

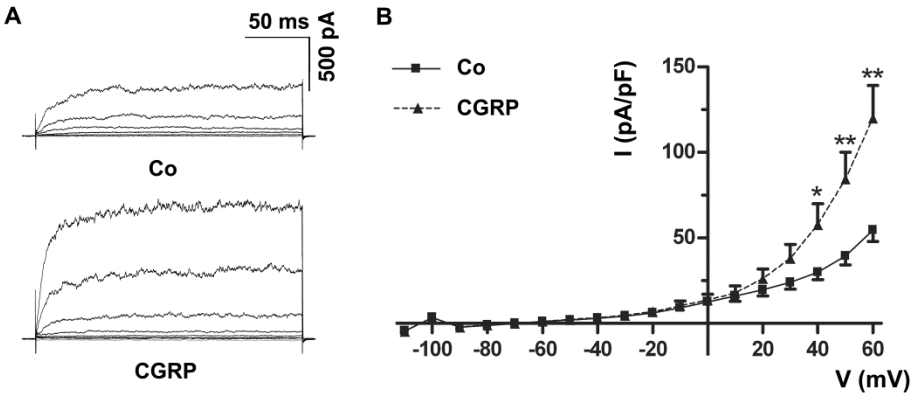

Figure 3. Influence of CGRP on I_K. A. Representative current traces from a myocyte in control conditions (Co) and in the presence of CGRP (10 nM) B. Mean normalized current (I)-V plot showing the influence of CGRP (10 nM). Each point represents the mean + s.e.mean from eight cells. * and ** denote statistically significant from control value at $p < 0.05$ and $p < 0.02$, respectively.

In a next series of experiments, cells were pre-exposed to either 1 mM TEA or to the more selective BK_{Ca} channel blocker iberiotoxin (100 nM). Both substances significantly decreased BK_{Ca} current, as also reflected by the smoother (less noisy) appearance of the current traces in the presence of the inhibitors (Figure 4A and 4C). In the presence of TEA, the mean I_K at +60 mV decreased from 22.6 $\pm$ 4.6 to 9.3 $\pm$ 2.0 pA/pF. Application of iberiotoxin decreased the I_K at +60 mV from 31.7 $\pm$ 7.3 to 23.4 $\pm$ 8.0 pA/pF. In the continuous presence of either TEA or iberiotoxin, the additional application of CGRP had no effect (Figure 4).

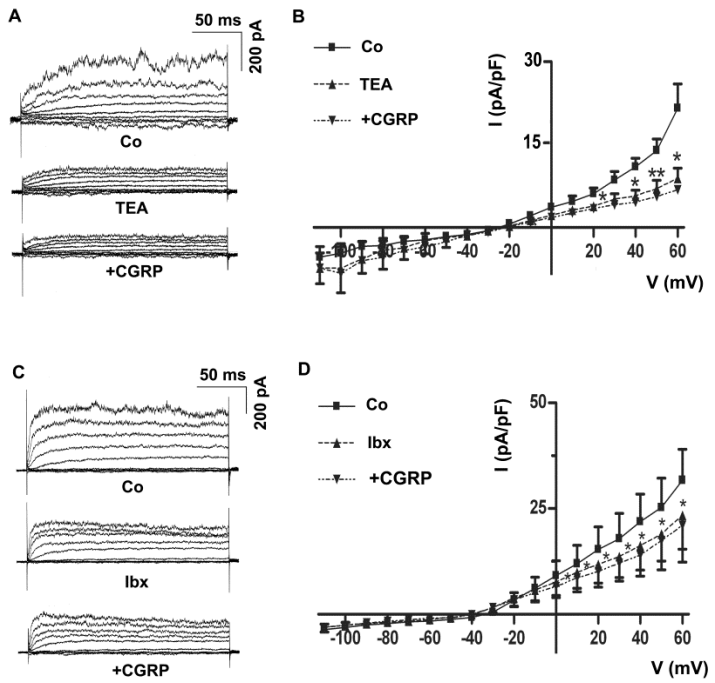

Figure 4. Influence of CGRP after inhibition of BKCa channels A. Representative current traces from a myocyte in control conditions (Co), in the presence of TEA (1 mM) and after extra addition of CGRP (10 nM). B. Mean normalized current (I)-V plot showing the effect of TEA and of additional exposure to CGRP. Each point represents the mean + s.e.mean from three cells. C. Representative current traces from a myocyte in control conditions (Co), in the presence of iberiotoxin (Ibx, 100 nM) and after extra addition of CGRP (10 nM). D. Mean normalized current (I)-V plot showing the effect of iberiotoxin and of additional exposure to CGRP. Each point represents the mean + s.e.mean from five cells. * and ** denote statistically significant from control value at $p < 0.05$ and $p < 0.02$, respectively.

In six cells, the influence of addition of CGRP was investigated in the presence of the inhibitor of K_{ATP} channels, glibenclamide (10 µM). Pre-exposure to glibenclamide did not influence control currents. In the presence of glibenclamide, CGRP increased the mean current at +60 mV from 15.8 $\pm$ 3.2 to 25.3 $\pm$ 6.4 pA/pF (Figure 5A), i.e. to 160 % of control.

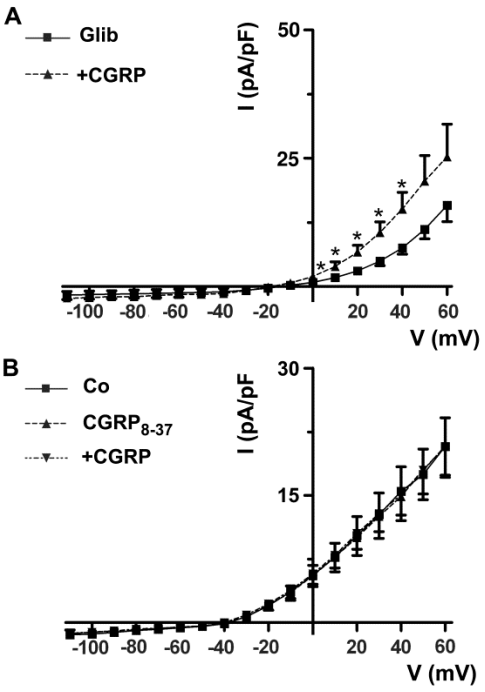

Figure 5. Influence of inhibition of KATP channels and the CGRP receptor. A. Mean normalized current (I)-V plot showing the effect of CGRP in the presence of glibenclamide (Glib, 10 :M). B. Mean normalized current (I)-V plot showing the lack of effect of CGRP in the presence of CGRP8-37. (100 :M). Each point represents the mean + s.e.mean from four (A) or five (B) cells. * denotes statistically significant from control value at $p < 0.05$.

CGRP acts by stimulating the CGRP receptor and the adenylyl cyclase pathway

In order to verify whether the effect of the neuropeptide is receptor-mediated, the CGRP receptor antagonist $CGRP_{8-37}$ was applied. The mean I-V relationships obtained in five cells are shown in Figure 5B. Pre-exposure to 100 nM $CGRP_{8-37}$ completely prevented the current potentiation by CGRP.

In five cells, the influence of CGRP was tested after pre-exposure to 100 µM SQ22536, an adenylyl cyclase inhibitor. The compound significantly decreased outward K^+ currents in the +40 to +60 mV range (Figure 6A). The mean I_K at +60 mV decreased from 31.5 $\pm$ 4.6 to 21.0 $\pm$ 2.9 pA/pF. A direct influence of SQ22536 on BK_{Ca} channels was excluded since pre-exposure to iberiotoxin did not block the effect of SQ22536 (Figure 6B). In the continuous presence of SQ22536, CGRP had no effect (Figure 6A). Currents in the combined presence of SQ22536 and CGRP were not significantly different from currents in the presence of SQ22536 alone.

In five cells, the influence of the protein kinase A (PKA) inhibitor Rp-cAMPS (10 µM) was tested. The compound significantly decreased control currents (Figure 6C). A direct influence of Rp-cAMPS on BK_{Ca} channels was excluded since pre-exposure to iberiotoxin did not block the influence of Rp-cAMPS (Figure 6D). Furthermore, after pre-exposure to Rp-cAMPS, CGRP failed to increase K^+ currents (Figure 6C). Currents in the combined presence of Rp-cAMPS and CGRP were not significantly different from currents in the presence of Rp-cAMPS alone.

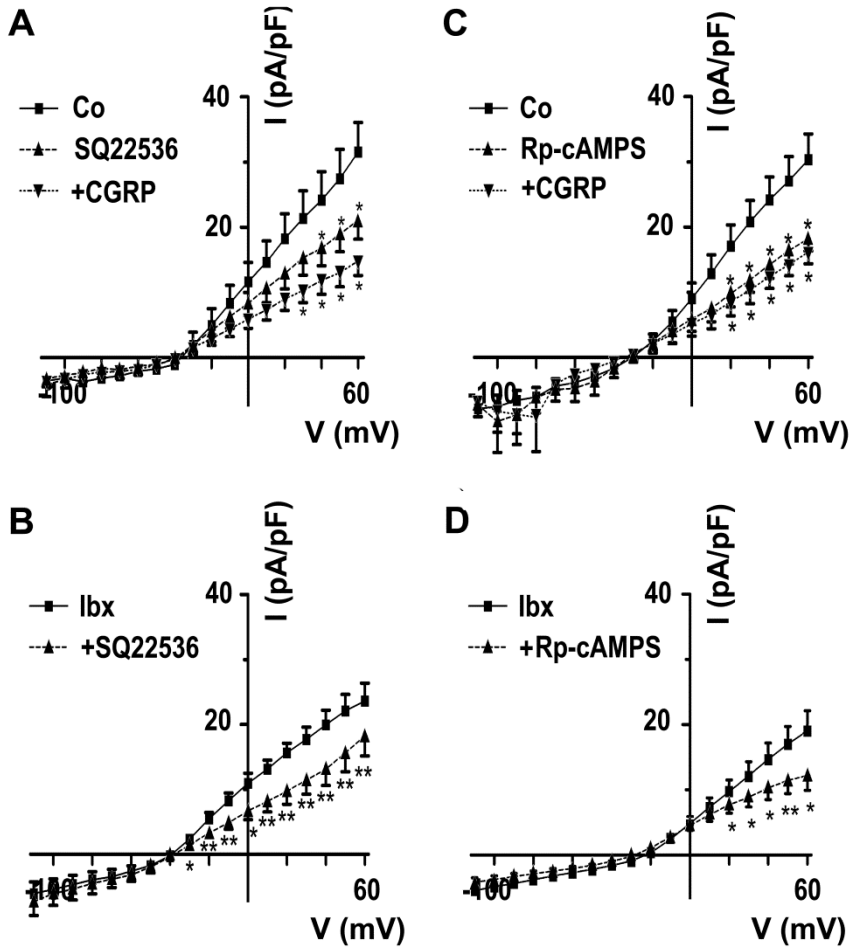

Figure 6. Influence of inhibition of adenylyl cyclase or PKA. A. Mean normalized current (I)-V plot showing the influence of CGRP (10 nM) after pre-exposure to SQ22536 (100 µM). B. Mean normalized I-V plot showing the influence of SQ22536 (100 µM) after inhibition of BK_{Ca} channels with iberiotoxin (Ibx, 100 nM). C. Mean normalized I-V plot showing the influence of CGRP (10 nM) after pre-exposure to Rp-cAMPS (10 µM). D. Mean normalized I-V plot showing the influence of Rp-cAMPS (10 µM) after inhibition of BK_{Ca} channels with iberiotoxin (Ibx, 100 nM). In A, B, C, and D, each point represents the mean + s.e.mean from 5 cells. * and ** denote current value statistically significant from the control value (A,C) or current in the presence of Ibx (B,D) at $p < 0.05$ or $p < 0.01$, respectively.

2. INFLUENCE OF METHANANDAMIDE AND CGRP ON MEMBRANE POTENTIAL

Some isolated resting myocytes were current clamped at 0 pA to measure membrane potential. Typical traces are shown in Figure 7. In three cells, the application of methanandamide (10 µM) failed to alter the membrane potential (-35.5 $\pm$ 4.8 mV five min after addition of the cannabinoid, vs -35.2 $\pm$ 4.3 mV in control conditions). Conversely, the addition of CGRP (10 nM) slowly hyperpolarized the membrane potential from -30.4 $\pm$ 2.3 mV to -37.2 $\pm$ 3.2 mV as measured five min after application of the neuropeptide (cf. Figure 7) .

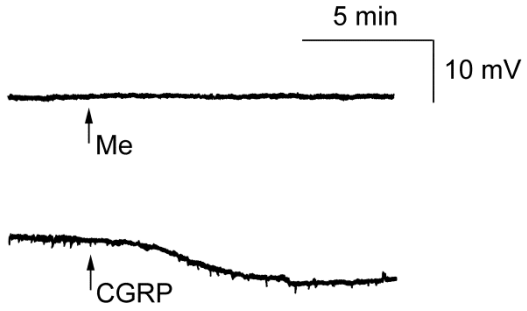

Figure 7. Influence of methanandamide (Me, 10 µM) and CGRP (10 nM) on the membrane potential of isolated myocytes, as measured in current clamp mode.

DISCUSSION

The present findings show that the cannabinoid methanandamide fails to increase membrane K^+ currents and fails to hyperpolarize the membrane potential of isolated smooth muscle cells of small mesenteric arteries. Moreover, they reveal that the neuropeptide CGRP is acting specifically and directly on these cells by increasing the BK_{Ca} channel activity in a receptor-, cAMP- and PKA-dependent way (Figure 8). This is consistent with earlier studies suggesting that methanandamide relaxes and hyperpolarizes intact rat mesenteric vessels by releasing the neuropeptide from perivascular nerves.

In the present study, the stable cannabinoid derivative methanandamide was found to decrease K^+ current in isolated smooth muscle cells from small mesenteric arteries. A similar influence has been described for smooth muscle cells isolated from the aorta of the rat (Van den Bossche and Vanheel 2000). In the cited study, detailed investigations using the blockers TEA and 4-aminopyridine have shown a direct influence of cannabinoids on the delayed rectifier K^+ channels, independent from their possible interaction with cannabinoid receptors. In the present study, neither iberiotoxin nor the combined presence of TEA (1 mM) and glibenclamide were able to affect the influence of methanandamide, while it was completely abrogated after pre-exposure to 4-AP. We propose, therefore, a similar inhibition of the delayed rectifier in mesenteric artery myocytes, although this was not further investigated.

It has been shown that the mechanism of vasorelaxation to cannabinoids differs between conduit and resistance mesenteric arteries (O'Sullivan *et al.* 2004). Moreover, in a previous

study, we have shown that anandamide and methanandamide did not notably alter membrane potential of the smooth muscle of the main mesenteric artery, while the cannabinoids caused a substantial and endothelium-independent hyperpolarization in small mesenteric arteries (Vanheel and Van de Voorde 2001). The present findings, in which a similar inhibition of K^+ current was found in smooth muscle cells from resistance arteries as was described for aortic smooth muscle cells, strongly argue against a direct hyperpolarizing influence of methanandamide on the smooth muscle cells from small mesenteric arteries. In the previous microelectrode study, the anandamide-induced hyperpolarization was insensitive to the classical CB1 receptor antagonist SR141716A, but fully blocked by capsazepine (Vanheel and Van de Voorde 2001), suggesting that the substance acts indirectly by stimulation of TRPV1 receptors on perivascular sensory nerves with the subsequent release of the vasodilator neurotransmitter CGRP (Zygmunt *et al.* 1999, Ralevic *et al.* 2000, Vanheel and Van de Voorde 2001). The present membrane potential measurements in isolated myocytes fully confirm such an indirect action.

The application of exogenous CGRP substantially increased mesenteric smooth muscle cell I_K. Moreover, the membrane potential of isolated myocytes substantially hyperpolarized after exposure to CGRP. These findings unequivocally show that the neuropeptide is acting directly on the smooth muscle cells and that at least part of the hyperpolarization we previously found in intact small mesenteric arteries (Breyne and Vanheel 2006) is endothelium-independent. This observation is also in line with literature data showing that CGRP-induced vasorelaxation of rat mesenteric arteries is endothelium-independent (Takenaga *et al.* 1995, Gao *et al.* 1996).

A number of studies have shown that CGRP increases cAMP content in vascular smooth muscle (Kageyama *et al.* 1993, Yoshimoto *et al.* 1998). cAMP-dependent protein kinase may activate K_{ATP} channels, as in pig coronary arteries (Wellman *et al.* 1998) and rabbit mesenteric artery (Quayle *et al.* 1994), or BK_{Ca} channels, as in pig coronary arteries (Minami *et al.* 1993) and rat aorta (Sadoshima *et al.* 1988) and tail arteries (Schubert *et al.* 1996, Schubert and Nelson 2001). In some vessels, the CGRP-induced vasodilation was mediated by activation of both BK_{Ca} and K_{ATP} channels (Hong *et al.* 1996, Herzog *et al.* 2002). In preconstricted rat coronary arteries, charybdotoxin inhibited the CGRP-induced vasorelaxation, while in the resting arteries only the pretreatment with a combination of

glibenclamide and charybdotoxin attenuated the CGRP-induced decrease in $[Ca^{2+}]_i$ and tension (Sheykhzade and Nyborg 2001). In the present experiments, we found low concentrations of TEA and the presence of the more selective inhibitor of BK_{Ca} channels, iberiotoxin, to fully block the influence of CGRP. It was also inhibited by the CGRP receptor antagonist $CGRP_{8-37}$, as well as by the adenylyl cyclase inhibitor SQ22536 and the PKA inhibitor Rp-cAMPS. Moreover, the K_{ATP} channel inhibitor glibenclamide had no effect. This shows that in the present conditions CGRP induces BK_{Ca} channel activation of rat mesenteric artery smooth muscle cells in a receptor-, cAMP- and PKA-dependent way.

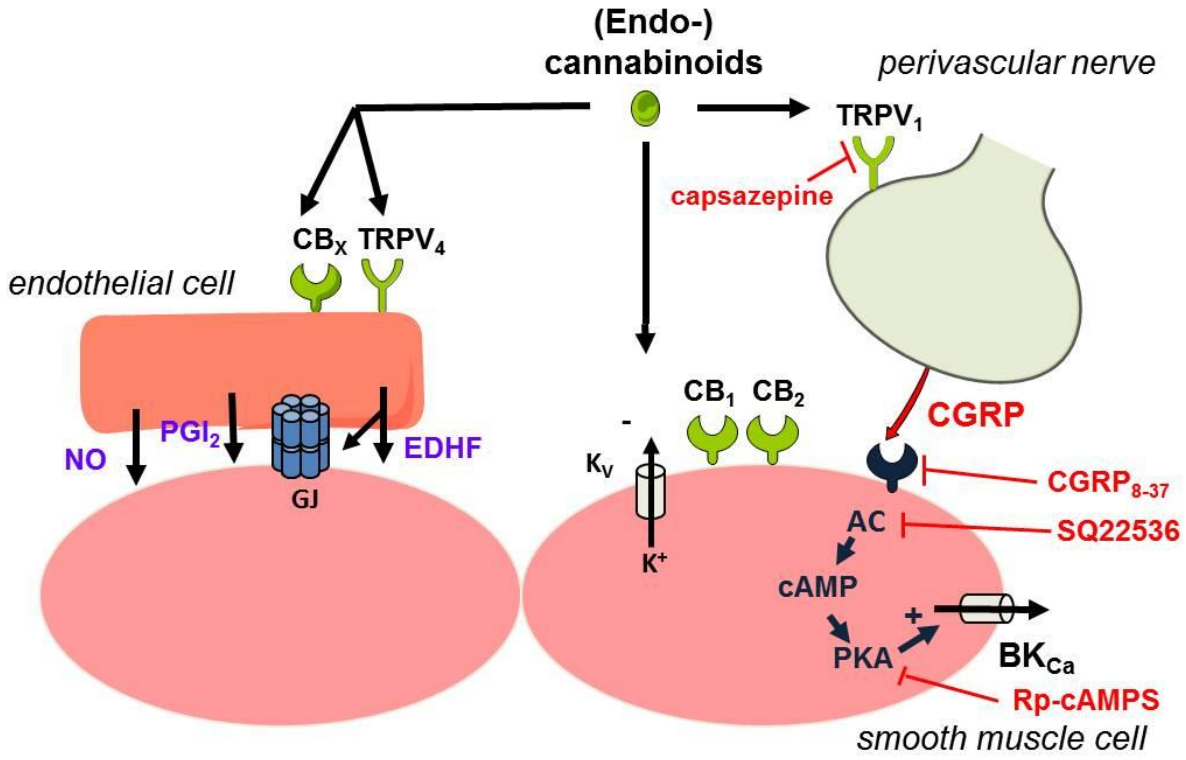

Figure 8. Findings of the present study as related to possible mechanisms for cannabinoid induced vasorelaxation. Cannabinoids may activate classical CB1 or CB2 receptors of the vascular smooth muscle cells. Various cannabinoids have also been shown to activate non CB1/non CB2 (CBx) receptors and TRPV4 receptors of the endothelial cells, causing the release of the endothelial relaxing factors NO and PGI2 and activating the EDHF pathway. In addition, activation of TRPV1 receptors on perivascular nerves has been shown, stimulating the release of vasodilator neuropeptides such as CGRP. In the present study, it is concluded that the cannabinoid methanandamide decreases delayed rectifier (Kv) currents of small mesenteric artery smooth myocytes and that CGRP causes BKCa activation in a receptor-, cAMP- and PKA-dependent way.

CONCLUSION

In summary, we have shown that exogenous CGRP potently activates BK_{Ca} currents in small mesenteric artery smooth muscle cells, and substantially hyperpolarizes the membrane potential of these cells. The influence on BK_{Ca} current is receptor-dependent and mediated by adenylyl cyclase and PKA. Moreover, methanandamide failes to hyperpolarize the membrane potential of these myocytes and decreases K^+ currents in a way similar as has been described for smooth muscle cells of the aorta. The latter finding is fully consistent with studies reporting that the cannabinoid hyperpolarizes and relaxes intact mesenteric arteries indirectly, by releasing CGRP from perivascular sensory nerves.

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The authors declare that they have no conflicts of interest.

Chapter VI: Connexin hemichannels contribute to Ca^{2+} dynamics and contractility of smooth muscle cells in small mesenteric arteries

Connexin hemichannels contribute to Ca^{2+} dynamics and contractility of smooth muscle cells in small mesenteric arteries

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In preparation

Abstract

Rationale: Gap junctions are important for coordinating vascular cell function and controlling vessel diameter and blood flow. Gap junctions are formed by the interaction of two connexin hemichannels from each of the connecting cells. Hemichannels may also reside as non-junctional closed hemichannels in the plasma membrane that can be opened by various stimuli leading to the entry/loss of ions and the escape of ATP. Interestingly, hemichannels form a Ca^{2+} entry pathway that is also controlled by the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$).

Objective: We hypothesized that Ca^{2+}-controlled Ca^{2+} entry and ATP release *via* hemichannels might contribute to smooth muscle cell (SMC) Ca^{2+} dynamics, thereby possibly influencing blood vessel contractility. We used various tools inhibiting or promoting connexin hemichannel opening to investigate their influence on SMC Ca^{2+} dynamics and blood vessel contractility.

Methods and Results: Small mesenteric arteries from rat showed Cx37 and Cx43 expression in SMCs, without evidence for Panx1. Exposure of mesenteric arteries to norepinephrine induced Ca^{2+} oscillations in SMCs and vessel contraction that were inhibited by the connexin channel blockers carbenoxolone and connexin mimetic peptide Gap27, and suramin and PPADS as purinergic receptor antagonists. Ca^{2+} oscillations were also inhibited by TAT-L2 peptide that blocks Ca^{2+}-promoted opening of Cx43 hemichannels and by TAT-CT9 peptide that prevents closure of Cx43 hemichannels at micromolar $[Ca^{2+}]_i$. Most notably, TAT-L2 strongly inhibited norepinephrine-induced contraction.

Conclusions: These results indicate that Cx43 hemichannels play a role in SMC Ca^{2+} oscillations and contractility, and bring up a novel handle to exert control over vascular tone.

INTRODUCTION

Vascular function is largely determined by the concentration of intracellular cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in the endothelial and smooth muscle cells (SMCs) of the blood vessel wall. The steady, baseline level of $[Ca^{2+}]_i$ determines the contraction state of SMCs and superimposed on this are changes in $[Ca^{2+}]_i$ that are organized in time (Ca^{2+} oscillations) and space (Ca^{2+} waves) and play an essential role in determining vascular tone (Ruehlmann *et al.* 2000, Savineau and Marthan 2000).

Gap junction channels composed of connexin proteins form a direct communication pathway between vascular cells that are crucial in coordinating $[Ca^{2+}]_i$ signals and vessel function, setting the balance between vasodilation and vasoconstriction. For example, cyclic variations of the vascular diameter, called vasomotion, are induced by synchronous Ca^{2+} oscillations of SMCs in which gap junctions are involved (Koenigsberger *et al.* 2005). Before being incorporated into gap junctions, unapposed/non-junctional connexin hemichannels reside in the plasma membrane in a closed state. Recent evidence suggests that non-junctional hemichannels can be opened by various messengers and conditions, thereby providing a conduit that allows the passage of ions and messengers like ATP or other substances with MW up to 1.5 kDa (Kang *et al.* 2008). Connexin hemichannels are Ca^{2+}-permeable channels that can mediate Ca^{2+} entry (Saez *et al.* 2010, Sanchez *et al.* 2010, Schalper *et al.* 2010, Fiori *et al.* 2012). Moreover, their opening is also influenced by $[Ca^{2+}]_i$ in a biphasic manner (de Vuyst *et al.* 2006, De Vuyst *et al.* 2009, Ponsaerts *et al.* 2010, De Bock *et al.* 2012, Wang *et al.* 2012): once open, Ca^{2+} entry further elevates $[Ca^{2+}]_i$, favouring more hemichannel opening in a positive feedback loop. Once $[Ca^{2+}]_i$ attains levels above ~500 nM, Ca^{2+} inhibits hemichannel opening, providing negative feedback with subsequent hemichannel closure and stop of this Ca^{2+} entry pathway. The biphasic Ca^{2+}-dependence of hemichannels greatly resembles the Ca^{2+}-dependence of inositol-trisphosphate receptor (IP_3R) channels that are Ca^{2+} release channels in the ER/SR that are central in generating Ca^{2+} oscillations in diverse cell types including SMCs (Morel *et al.* 2003, Dupont *et al.* 2007, Bai *et al.* 2009). Based on this similarity, we hypothesized that connexin hemichannels may contribute to Ca^{2+} oscillations.

Vascular cells express several connexins, including Cx37, Cx40 and Cx43 in endothelial cells, and Cx43 and Cx37 in SMCs (Haefliger *et al.* 2004). Pannexins are related to the connexin

protein family: they have a similar topology but little sequence homology with connexins and form plasma membrane channels that, like connexin hemichannels, are Ca^{2+}-permeable and may function as ATP release channels (D'Hondt *et al.* 2011). Recent work has demonstrated that Panx1, which is expressed in SMCs of resistance arteries in several vascular beds (Lohman *et al.* 2012), plays a role in the vasoconstriction induced by α1-adrenergic stimulation of thoracodorsal arteries at least in mice (Billaud *et al.* 2011, Billaud *et al.* 2012).

The purpose of the present work was to determine whether connexin hemichannels are involved in SMC Ca^{2+} oscillations and control of vascular tone. We investigated $[Ca^{2+}]_i$ dynamics and vessel contractility in acutely isolated small mesenteric blood vessels of rats exposed to the vasoconstriction-inducing agent norepinephrine. Besides classical modulators such as carbenoxolone and Gap27, we applied several synthetic peptides to specifically interfere with Cx43 hemichannel function, either inhibiting or promoting their function. The synthetic L2 peptide consists of amino acid sequence 119 to 144 of the cytoplasmic loop of Cx43. This peptide prevents intramolecular interactions between the C-terminal tail (CT) and the cytoplasmic loop (CL) of Cx43, thereby inhibiting hemichannel ATP release (Ponsaerts *et al.* 2010, D'Hondt *et al.* 2013) while slightly favoring the open state of gap junctions (Seki *et al.* 2004). Another peptide, CT9, that corresponds to the last 9 amino acids of the Cx43 C-terminal was used in this study. A similar 10 amino acid long peptide was found to stimulate hemichannel opening/ATP release by preventing hemichannel closure at high (1 µM) $[Ca^{2+}]_i$ (Ponsaerts *et al.* 2010).

METHODS

Small mesenteric artery preparation

Small mesenteric arteries from female Wistar rats (180-280 g) were used. The experiments were approved by the ethical committee on animal research of Ghent University and conform the directive 2010/63/EU of the European Parliament. The animals were anaesthetised by a lethal dose (200 mg/kg) of pentobarbitone and killed by cervical dislocation.

Polymerase chain reaction

In these experiments, the endothelium was removed by bubbling the lumen of the vessels for 2 min with a 95% O_2 – 5% CO_2 gas mixture. Total RNA from small mesenteric artery smooth muscle was isolated using RNeasy Plus mini kit (Qiagen). Reverse transcription was performed by iScriptcDNA synthesis kit (BioRad) and cDNA was amplified using Taq DNA polymerase kit (Invitrogen). Primer sequences were: Cx37 forward 5'-AGCTCTGCATCCAAGAAGCAGTA-3' and reverse 5'-AGTTGTCTCTCAAGTGCCTTTGA-3'; Cx43 forward 5'-GAGATGCACCTGAAGCAGATTGAA-3' and reverse 5'-GATGTTCAAAGCGAGAGACACCAA-3'; Panx1 forward 5'-TTCTTCCCCTACATCCTGCT-3' and reverse 5'-GGTCCATCTCTCAGGTCCAA-3'; GAPDH (internal control) forward 5'-ACCACAGTCCATGCCATCA-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR protocol contained 35 cycles of 94 °C (45 sec), 50 °C (1 min) and 72 °C (1 min), preceded by 4 min 94 °C and followed by 5 min 72 °C. As a negative control, a sample lacking reverse transcriptase (RT-) was used. The PCR end products were separated on a 2 % agarose gel and visualized with ethidium bromide (Invitrogen).

Immunocytochemistry

SMCs from small mesenteric arteries were dissociated as previously described (Van den Bossche and Vanheel 2000) and immunocytochemistry was performed with antibodies for Cx43 and Cx37.

SMC isolation: artery segments were transferred to cold (4 °C) low calcium dissociation medium and were allowed to rest in this medium for 30 min. The small pieces were incubated with papain (17 U/ml), bovine serum albumin (1.98 mg/ml) and dithiothreitol (6.7 mM) for 20 min and afterwards for another 10 min with hyaluronidase (330 U/ml) and collagenase (410 U/ml). Cells were triturated and were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBSD) (10 min, room temperature) and subsequently permeabilized with 0.2 % Triton X-100 in PBSD (10 min, room temperature). Preincubation with 0.1 M glycine in PBSD for 1 h was done to prevent autofluorescence. Cells were incubated for 1 h with primary antibody (rabbit polyclonal anti-rat Cx43 (1/10000, Sigma, Belgium) or Cx37 (1/250, Zymed, Life Techologies, Belgium)) diluted in blocking solution

(0.4 % gelatine in PBSD), washed three times with PBSD and incubated for 1 h with the secondary antibody coupled to an Alexafluorophore 488 (goat anti-rabbit IgG antibody; Molecular Probes, 1/500). Cell nuclei were stained with DAPI (1µg/ml) and the preparations were then mounted in Vectashield (Vector Laboratories) to prevent photobleaching. Images were acquired on a Nikon TE300 inverted microscope with a x100 oil immersion objective (Fluor, NA 1.3, Nikon Benelux, Belgium).

Immunohistochemistry

Paraffin slices of small mesenteric arteries were deparaffined and an antigen retrieval solution was used (Lab Consult, Belgium). Preincubation with a blocking buffer (BSA, 50 mg; bovine skin gelatin, 500 µl; Triton X-100, 25 µl; PBS, 9 ml) for 30 min at room temperature was done. Preparations were incubated overnight at 4 °C with primary antibody anti-Cx43 polyclonal AB (1/250, Sigma, Belgium) or anti-Cx37 (1/100, Zymed, Life Techologies, Belgium) or anti-Panx1 polyclonal (1/1000, Santa Cruz Biotechnology, Germany) diluted in blocking solution. After washing, secondary antibody conjugated to alexa-594 (1/500, Molecular Probes, Life Technologies, Belgium) was added. The preparations were mounted with Vectashield + DAPI (Vector Labs, Lab Consult, Belgium). Images were acquired on a Nikon TE300 inverted microscope with a x20 objective (Plan Apo, NA 0.75, Nikon Benelux, Belgium).

Fluorescence recovery after photobleaching (FRAP)

SMC isolation was as described for immunocytochemistry (see above) and was followed by gentle trituration with a wide-bore Pasteur pipette to obtain small cell groups, including cell pairs or cell triplets. Cells were then loaded with 5-CFDA-AM (10 µM) in HBSS-HEPES for 1 h at room temperature, followed by 30 min de-esterification. Cells were transferred to a custom developed real-time laser scanning microscope (Leybaert *et al.* 2005) built on a Nikon Eclipse TE300 (Analis, Ghent, Belgium) with a x40 oil immersion objective (CFI Plan Fluo, Nikon – 1.4 NA) and a 488 nm laser excitation source (Cyan CW Laser, 488 nm - 100 mW, Newport Spectra-Physics, Utrecht, The Netherlands). After 1 min of recording, the cell in the middle of the field was photobleached by spot exposure (1 s) to increased power of

the 488 nm laser and fluorescence recovery, caused by dye influx from neighbouring non-bleached cells, was recorded over a 5 min period. The fluorescence recovery trace was then analyzed for the recovery of the signal expressed relative to the starting level before photobleaching.

Ca^{2+} imaging

Vessel segments were cannulated with a thin glass capillary (borosilicate glass, Hilgenberg, Malsfeld, Germany) with flame polished tip that was inserted into the lumen over the entire segment length. The diameter of the glass capillary was slightly (~15 %) larger than the vessel lumen diameter and cannulation was done to stabilize the preparation against possible smooth muscle contractions after challenging with norepinephrine. Visual inspection on the confocal microscope showed that by cannulating the vessel, most of the endothelium was removed since endothelial cells, visualised by the fluorescent Ca^{2+} indicator dye (see below) did not appear in most preparations.

$[Ca^{2+}]_i$ imaging was done with the fluorescent Ca^{2+} probe fluo-3 in combination with laser scanning confocal microscopy (Leybaert *et al.* 2005) built on a Nikon Eclipse TE300 (Analis, Ghent, Belgium) with a x40 oil immersion objective (CFI Plan Fluo, Nikon – 1.4 NA) and a 488 nm laser excitation source (Cyan CW Laser, 488 nm - 100 mW, Newport Spectra-Physics, Utrecht, The Netherlands). Excitation was done with 488 nm light (Cyan CW Laser, 488 nm - 100 mW, Newport Spectra-Physics, Utrecht, The Netherlands); the dichroic mirror was a dual-wavelength type (490-550DBDR) and the emission light was bandpass filtered at 522 nm (25 nm bandwidth – all filters from Omega Optical, Brattleboro, VT, USA – details can be found in Leybaert *et al.* (Leybaert *et al.* 2005)). Images of an optical section of the arterial wall (~123 µm x 93 µm and containing about 30-40 SMCs) were obtained at 2 frames per second and transferred directly to a PC equipped with an image acquisition and processing board (DT3155, Data translation, Marlboro, MA, USA). Off-line image analysis was done with custom-developed software (Fluoframes) written in Microsoft Visual C.

These experiments were done at room temperature. Cannulated arterial segments were loaded with 20 µM fluo-3-AM by incubation for 2 h at room temperature in HBSS-HEPES containing 0.05 % pluronic. The arteries were then washed in HBSS-HEPES, placed on a glass coverslip and immobilized by covering with a fine nylon mesh attached to a ring. The

coverslip with the cannulated vessel was transferred to the stage of the laser scanning inverted microscope and was continuously superfused with HBSS-HEPES at a rate of 1 ml/min. Point measurements in individual cells showed repetitive Ca^{2+} spikes and these spikes were quantified for frequency and amplitude. Only $[Ca^{2+}]_i$ peaks that were at least two standard deviations above the baseline noise were considered in the calculation of the spiking frequency and amplitude.

Tension measurements

Arterial segments (average diameter 311 ± 13 µm, n=86) were mounted into an organ bath of a small-vessel myograph, filled with 10 ml KRB solution for isometric tension measurements as described before (Van de Voorde and Vanheel 1997). In brief, two stainless steel wires (40 µm in diameter) were guided through the lumen of the segments. Each wire was fixed to a holder of the myograph: one holder was connected to a micrometer which was used to change the distance between the wires, the other holder was connected to a force-displacement transducer to measure the isometric tension changes. After mounting, the preparations were allowed to equilibrate for at least 30 min. The pretension settings were adjusted according to an optimal vessel lumen diameter that was calculated based on the relationship between passive wall tension and internal circumference (Mulvany and Halpern 1976). A first set of experiments was done at room temperature and with the endothelium removed by gas bubbling (as described under *Polymerase chain reaction*). In a subsequent set of experiments the endothelium was intact and the organ bath was heated to 37 °C. The presence of a functional endothelium was assessed by the ability of 10 µM acetylcholine to provoke more than 80 % relaxation of norepinephrine-induced tone. Control experiments showed that application of vehicle solutions had no effect on the norepinephrine-induced contractions.

Pharmacological agents

Norepinephrine bitartrate, carbenoxolone, suramin, pyridoxal-phosphate-6-azo(benzene-2,4-disulfonic-acid)tetra-sodium-salt (PPADS) and apyrase were purchased from Sigma-Aldrich (Bornem, Belgium). Fluo-3 acetoxymethyl ester (fluo-3-AM), 5-carboxyfluorescein-

diacetate-acetoxy-methylester (5-CFDA-AM) and pluronic acid F-127 were obtained from Molecular Probes (Invitrogen, Merelbeke, Belgium). Xestospongin C was purchased from Tocris (Bristol, UK).

Gap27 (SRPTEKTIFII) and scrambled Gap27 (TFEPIRISITK) were synthesized by Thermo Fisher Scientific (Ulm, Germany) at >80% purity. TAT-L2 (YGRKKRRQRRRDGANVDMHLKQIEIKKFKYGIEEHGK), TAT-L2 mutant (TAT-L2$^{H126K/I130N}$,YGRKKRRQRRRDGANVDMKLKQNEIKKFKYGIEEHGK), TAT-CT9 (YGRKKRRQRRRSRPRPDDLEI) and TAT-Cx43rev (YGRKKRRQRRRSIELDDPRPR) at >90% purity were synthesized by China Peptides (Shanghai, China). ^{10}panx1 (WRQAAFVDSY) was obtained from Lifetein (Edison, New Jersey, USA) and purity was > 90%.

Solutions and drugs

The KRB solution had the following composition (in mM): NaCl, 135; KCl, 5; $NaHCO_3$, 20; $CaCl_2$, 2.5; $MgSO_4.7H_2O$, 1.3; KH_2PO_4, 1.2; EDTA, 0.026; glucose, 10. The solution was gassed with 95% O_2 – 5% CO_2 (pH 7.4). The low calcium dissociation medium contained (mM): NaCl, 110; KCl, 5; $CaCl_2$, 0.16; $MgCl_2$, 2; KH_2PO_4, 0.5; EDTA, 0.49; NaH_2PO_4, 0.5; Taurine, 10; HEPES, 10; glucose, 10, titrated to pH 7.0. HBSS-HEPES contained (in mM): NaCl, 137; KCl, 5.36; $Na_2HPO_4.2H_2O$, 0.18; $CaCl_2.H_2O$, 0.95; $MgSO_4.7H_2O$, 0.81; KH_2PO_4, 0.44; HEPES, 25; glucose, 5.55 (pH 7.4, adjusted with NaOH). Phosphate-buffered saline (PBSD) contained (in mM): NaCl, 137; KCl, 2.68; $CaCl_2$, 0.90; $MgCl_2.6H_2O$, 0.334; KH_2PO_4, 1.47; $Na_2HPO_4.2H_2O$, 6.46 (pH 7.4).

Statistics

Results are expressed as means $\pm$ SEM. Statistical evaluation was performed using Student's t test or ANOVA, as appropriate. Values of $P<0.05$ were considered significantly different.

RESULTS

Connexin expression in rat small mesenteric arteries

PCR analysis demonstrated the presence of Cx37 and Cx43 in small mesenteric artery preparations (Figure 1A). Immunocytochemistry performed on isolated mesenteric artery SMCs (Figure 1B) or on slices of small mesenteric arteries (Figure 1C) confirmed the presence of Cx43 and Cx37. We found no evidence for Panx1 in SMCs of small mesenteric arteries, not at the mRNA level (Figure 1A) and not with immunohistochemistry (Figure 1C).

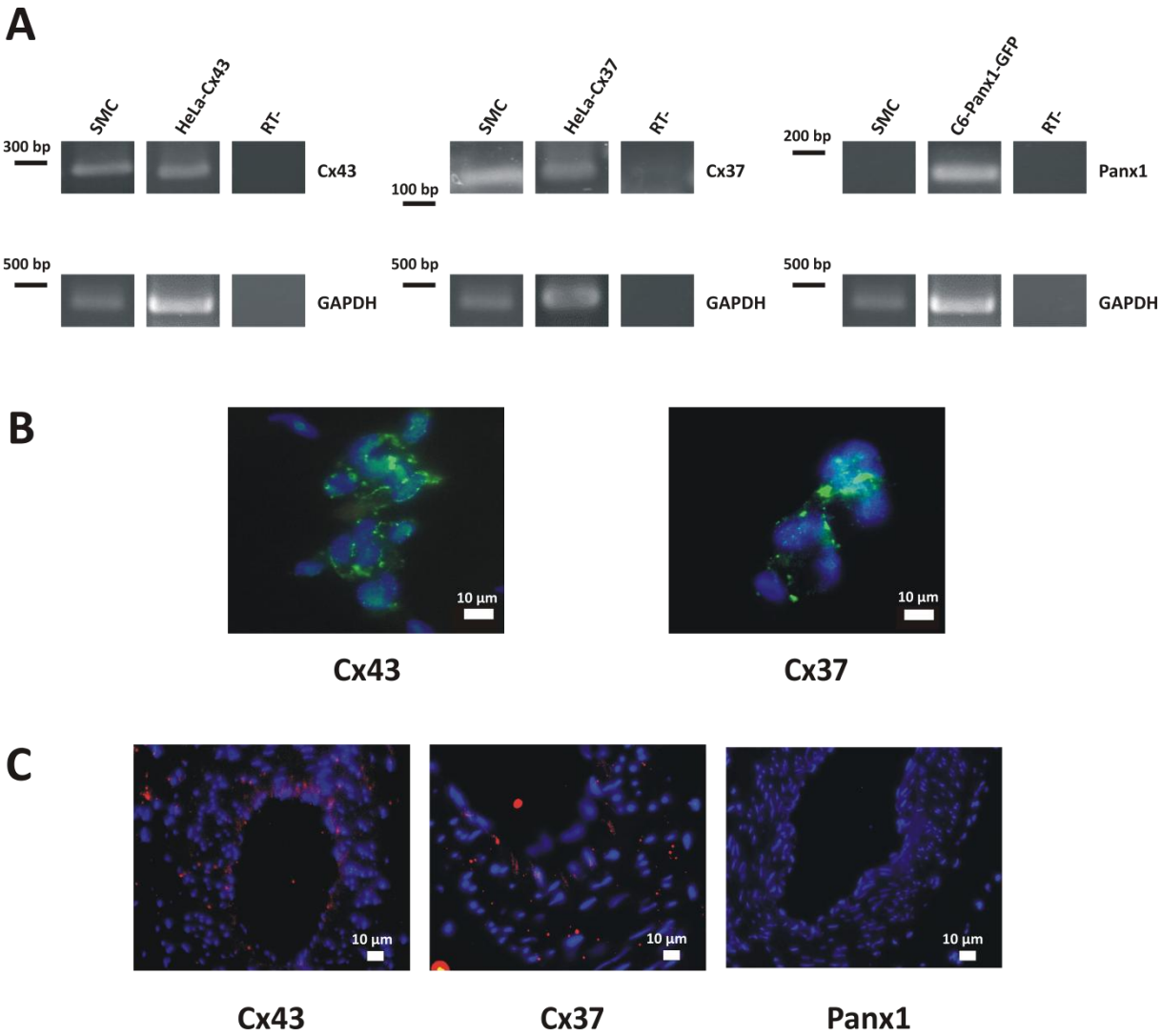


Figure 1. Connexins in small mesenteric artery SMCs. A. RT-PCR analysis illustrating presence of Cx43 and Cx37 mRNA transcripts and the absence of Panx1 mRNA transcripts in rat mesenteric SMCs. GAPDH was used as a control for cDNA synthesis. RT- is a reverse transcriptase negative control. B. Immunocytochemistry in isolated SMCs illustrating Cx43 and Cx37 expression (green); images overlayed on DAPI stainings (blue). C. Immunohistochemistry on vessel slices illustrating Cx43 and Cx37 expression (red) and absence of Panx1; images overlayed on DAPI staining (blue).

Norepinephrine triggers repetitive Ca^{2+} oscillations in rat mesenteric artery SMCs

We used norepinephrine to trigger Ca^{2+} oscillations. Norepinephrine (3 µM) triggered repetitive intracellular Ca^{2+} waves in SMCs of cannulated mesenteric arteries imaged with real-time confocal microscopy. Point measurements in individual cells showed repetitive Ca^{2+} spikes, further called Ca^{2+} oscillations (Figure 2A). Ca^{2+} oscillations were not synchronized between cells and vasomotion was never observed. Ca^{2+} oscillations induced by norepinephrine-only were recorded over an 11 min time window and during this period, the spiking rate (frequency) and amplitude decreased spontaneously (Figure 2A), as reported by others and probably resulted from desensitization to the agonist by continued exposure. On average, the Ca^{2+} oscillation frequency attained 2.51 $\pm$ 0.53 spikes per minute measured over the last 3 minutes of norepinephrine exposure (n=4, $p<0.05$ compared to exposure to solutions without norepinephrine where the frequency was zero). In the experiments that follow, we investigated the effect of several agents on the spiking frequency.

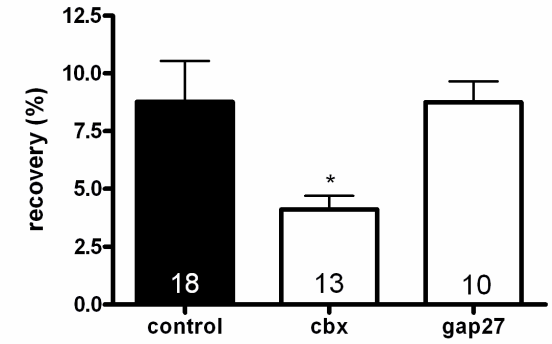
Norepinephrine-triggered Ca^{2+} oscillations involve IP_3R activation

Ca^{2+} oscillations are based on an increase of IP_3 in combination with positive and negative feedback of $[Ca^{2+}]_i$ on the IP_3R (Dupont *et al.* 2007). Xestospongin C (10 µM), a small-molecular-weight membrane-permeable inhibitor of IP_3Rs, had a drastic effect on the spiking activity as can be appreciated from the example trace in figure 2B. It significantly reduced the oscillation frequency of SMCs to 0.06 $\pm$ 0.03 spikes/min measured over the last 3 minutes (n = 8; $p<0.001$ compared to control frequency). Figure 3 summarizes average data on the spiking frequency expressed in a normalized manner, with the control response to norepinephrine set to 100 %. We next tested dantrolene (25 µM) to investigate the effect of ryanodine receptor (RyR) inhibition. These experiments showed no significant effects on oscillation frequency or amplitude (Figure 2C and Figure 3).

Interfering with connexin channel function inhibits norepinephrine-induced Ca^{2+} oscillations

We tested several connexin-targeting substances to determine whether these could influence norepinephrine-induced SMC Ca^{2+} oscillations. Addition of the non-selective connexin channel blocker, carbenoxolone (50 µM) during norepinephrine-induced Ca^{2+} spiking reduced the oscillation frequency to 0.02 $\pm$ 0.02 spikes/min (n = 4; p<0.001 comparison of raw frequency data – comparisons on normalized data see figure 3) (Figure 2D).

Gap27 composed of a conserved sequence of 11 amino acids in the second extracellular loop of both Cx37 and Cx43 rapidly blocks hemichannels (Wang *et al.* 2012) and with some delay also gap junctions (de Vuyst *et al.* 2006, De Bock *et al.* 2011, De Bock *et al.* 2012). This peptide (200 µmM) reduced the Ca^{2+} oscillation frequency to 0.05 $\pm$ 0.03 spikes/min (n = 6; p<0.001) (Figure 2E and Figure 3). Control experiments on freshly isolated SMC pairs demonstrated that Gap27 (200 µM) had no effect on gap junctional dye coupling (measured with fluorescence recovery after photobleaching, FRAP) when applied for 60 min (supplementary figure 1). Scrambled Gap27 ($gap27^{scr}$) had no effect on the Ca^{2+} oscillation frequency (Figure 2F and Figure 3).



Supplementary figure 1. Dye coupling in isolated SMC pairs was studied with fluorescence recovery after photobleaching (FRAP). Average FRAP experiments showed a limited fluorescence recovery that was significantly inhibited by carbenoxolone (cbx, 50 µM, 20 min) but not by Gap27 (200 µM, 60 min). In line with previously reported observations that Gap27 only inhibits GJs upon longer exposures (Braet *et al.* 2003, Decrock *et al.* 2009, De Bock *et al.* 2011). The number of experiments is given in the bars. * p<0.05

Previous work has demonstrated that hemichannels contribute to Ca^{2+} oscillations by acting as a $[Ca^{2+}]_i$-controlled Ca^{2+} entry pathway (De Bock *et al.* 2012) We here used L2 and CT9 peptides (linked to a TAT permeation sequence) to inhibit the positive and negative feedback of $[Ca^{2+}]_i$ on hemichannel Ca^{2+} entry respectively (Figure 4 depicts the biphasic effect of $[Ca^{2+}]_i$ on hemichannel opening and the alterations induced by the synthetic peptides L2 and CT9), and tested their effect on norepinephrine-triggered Ca^{2+} oscillations in mesenteric artery SMCs. We applied peptides covalently linked to a TAT sequence to facilitate their plasma membrane passage and uptake into the cells. Addition of TAT-L2 (100 µM) or TAT-CT9 (100 µM) attenuated the Ca^{2+} oscillation frequency to 0.87 $\pm$ 0.27 spikes/min (n = 7; p<0.001) and 0.69 $\pm$ 0.22 spikes/min respectively (n = 5; p<0.001) (Figure 2GI and Figure 3). By contrast, mutant TAT-L2 peptide (TAT-$L2^{H126K/I130N}$) and reversed sequence TAT-CT9 (TAT-$CT9^{rev}$) had no effect on the Ca^{2+} oscillations (Figure 2HI and Figure 3). Figure 2I illustrates that TAT-CT9 not only inhibited the Ca^{2+} oscillations but also tended to increase baseline $[Ca^{2+}]_i$ towards the end of the 8 min exposure. In fact, such effect is expected because CT9 prevents hemichannel closure at high $[Ca^{2+}]_i$ and may thus effectively promote Ca^{2+} entry. We further analyzed baseline fluo-3 fluorescence over the last 4 min of the recordings (expressed relative to the fluorescence in the preceeding 4 min) and found that TAT-CT9 significantly increased the baseline signal as compared to control recordings, while TAT-$CT9^{rev}$ or TAT-L2 had no such effect (Table 1).

Billaud et al. (Billaud *et al.* 2011) reported a role for Panx1 in α1-adrenergic receptor-mediated vasoconstriction and we therefore tested ^{10}panx1 (200 µmol/L), a pannexin1 mimetic peptide that inhibits these channels (Wang *et al.* 2007). These experiments showed no significant effects on the oscillation frequency or amplitude (Figure 2K and 3).

Table 1. TAT-CT9 increases SMC baseline $[Ca^{2+}]_i$

Condition	Fluo-3 fluorescence last 4 min divided by fluorescence during preceding 4 min of $[Ca^{2+}]_i$ recording (%)
Control	96.2 $\pm$ 0.16 % (n=4)
TAT-CT9	122.7 $\pm$ 7.23 % (n=5)**
TAT-$CT9^{rev}$	97.6 $\pm$ 0.68 % (n=5)
TAT-L2	100.6 $\pm$ 0.52 % (n=7)

** p < 0.01 compared to all other conditions

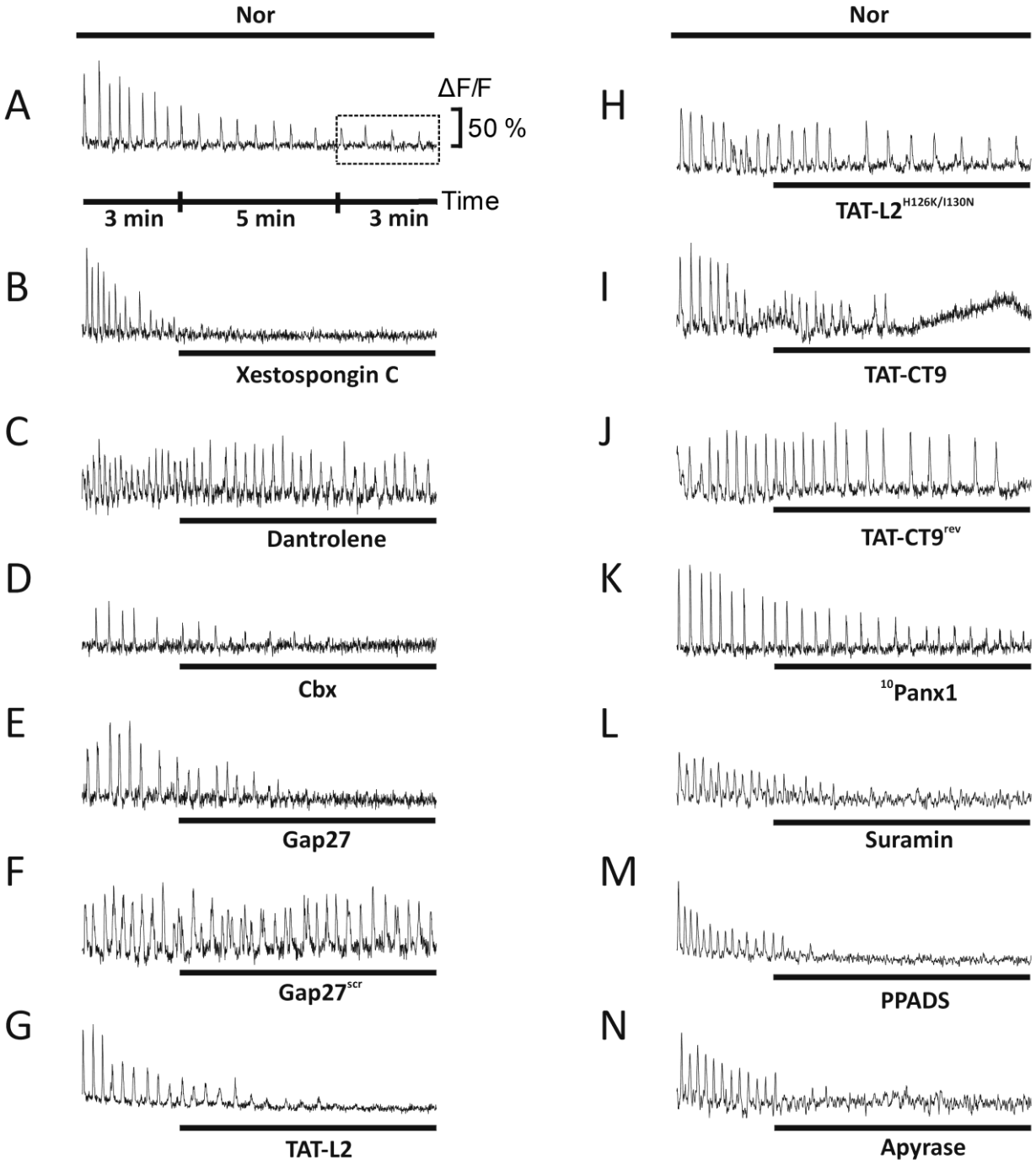


Figure 2. Example traces of Ca^{2+} oscillations induced by norepinephrine (Nor) in an individual small mesenteric artery SMC and the effect of various inhibitory/modulatory substances used. The rectangle in dotted line indicates the last 3 min time window used for frequency analysis. Traces A-N are discussed and referred to in the text.

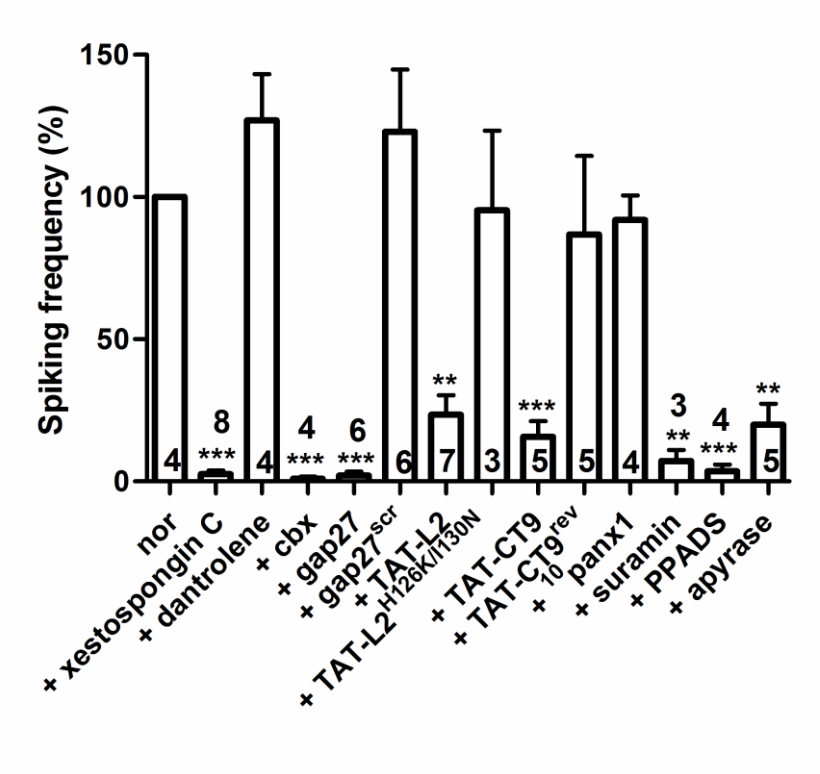


Figure 3. Average data of effects of various inhibitor/modulator substances on the oscillation frequency in the time window illustrated in figure 2 (control set to 100 %). The number of experiments is given in/above the bars. ** p<0.01 *** p<0.001.

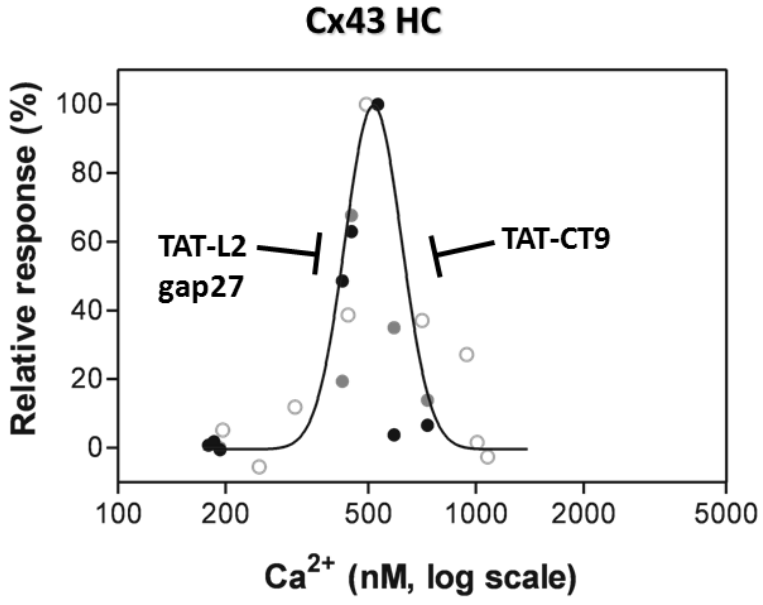


Figure 4. Ca^{2+} activation of hemichannels composed of Cx43 is characterized by a bell-shaped $[Ca^{2+}]_i$-dependence (hemichannel ATP release and dye uptake studies, derived from data in De Vuyst *et al.* 2009). Gap27 and TAT-L2 peptide remove the inclining phase at low $[Ca^{2+}]_i$, TAT-CT9 peptide removes the declining phase at $[Ca^{2+}]_i$ above 500 nM (Ponsaerts *et al.* 2010). Black dots: C6Cx43 ATP release; grey dots: C6Cx43 dye uptake; white dots: HeLaCx43 ATP release)

Purinergic receptor antagonists and ATP degrading enzymes inhibit norepinephrine-induced Ca^{2+} oscillations

Connexin hemichannel opening is associated with the release of messengers such as ATP. In order to test whether extracellular ATP plays a role in norepinephrine-induced Ca^{2+} spiking, we tested two purinergic receptor antagonists. Suramin (200 µM) and PPADS (75 µM), diminished the oscillation frequency to respectively 0.18 $\pm$ 0.10 and 0.09 $\pm$ 0.06 spikes/min (n = 3 and 4 respectively; $p<0.001$ for both) (Figure 2LM and Figure 3). We further applied enzymes known to degrade ATP. Apyrase V (5 U/ml) degrades ATP into ADP and apyrase VI (5 U/ml) further degrades ADP to AMP. In the presence of these two enzymes, the oscillation frequency was reduced to 0.50 $\pm$ 0.19 spikes/min (n = 5; $p<0.001$ compared to control frequency) (Figure 2N and Figure 3).

Carbenoxolone, Gap27, TAT-L2, PPADS and suramin also inhibit norepinephrine-induced contraction of rat small mesenteric arteries

Ca^{2+} oscillations are known to control blood vessel tone. In the following experiments we tested the effect of some of the compounds that had profound effects on Ca^{2+} oscillations, on the contractility as determined in isometric tension measurements in small mesenteric arteries. As the $[Ca^{2+}]_i$ measurements were performed on cannulated blood vessels, a procedure that largely removes the endothelium, we also removed the endothelium in the contraction experiments by bubbling the lumen of the vessels for 2 minutes with gas (95 % O_2 and 5 % CO_2). In a first series, measurements were performed at room temperature.

Figure 5A illustrates a control tension measurement experiment with contraction induced by norepinephrine (3 µM). Preincubation with carbenoxolone (50 µM, 20 min) inhibited the norepinephrine-induced contractions (Figure 5A). The area under the contraction curve (AUC) was decreased to ~56 % (average data see Figure 6). We also verified the peak amplitude (peak force) and the force averaged over the last 3 min of norepinephrine exposure. The peak force was decreased to ~60 % and the force during the last 3 minutes attained 53 % of the control values, i.e. these contraction parameters were influenced to a comparable extent as the AUC. Gap27 (200 µM, 20 min) and TAT-L2 (100 µM, 20 min) inhibited the norepinephrine-induced contractions (Figure 5BC) and decreased the AUC to

~74 % and ~50 % of control, the peak force to ~81 % and ~51 % and the force during the last 3 minutes of the recording to ~70 % and ~56 %. Surprisingly, TAT-CT9 had no effect on contractility while it inhibited Ca^{2+} oscillations (Figure 5D). Suramin (200 µM, 20 min) and PPADS (75 µmM, 20 min) inhibited the norepinephrine-induced contractions (Figure 5KL), reducing the AUC to 30 % and 67 % of control (Figure 6) while the peak force and the force during the last 3 minutes of the recording were reduced to ~26 % and ~70 %.

We repeated the tension measurements on blood vessels with an intact endothelium and performed the experiments at 37 °C instead of room temperature. Under those conditions carbenoxolone, Gap27, TAT-L2, PPADS and suramin again significantly inhibited the norepinephrine-induced contractions (Figure 5G-L and Figure 6). Importantly, the effects of these compounds were comparable with the findings at room temperature/without endothelium, and none of the measured AUC values (except TAT-L2), was significantly different from its corresponding measurement at room temperature/without endothelium. Remarkably, TAT-L2 had a significantly stronger effect at 37 °C/intact endothelium as compared to room temperature/no endothelium, reducing the AUC to ~20 % of control (Figure 6).

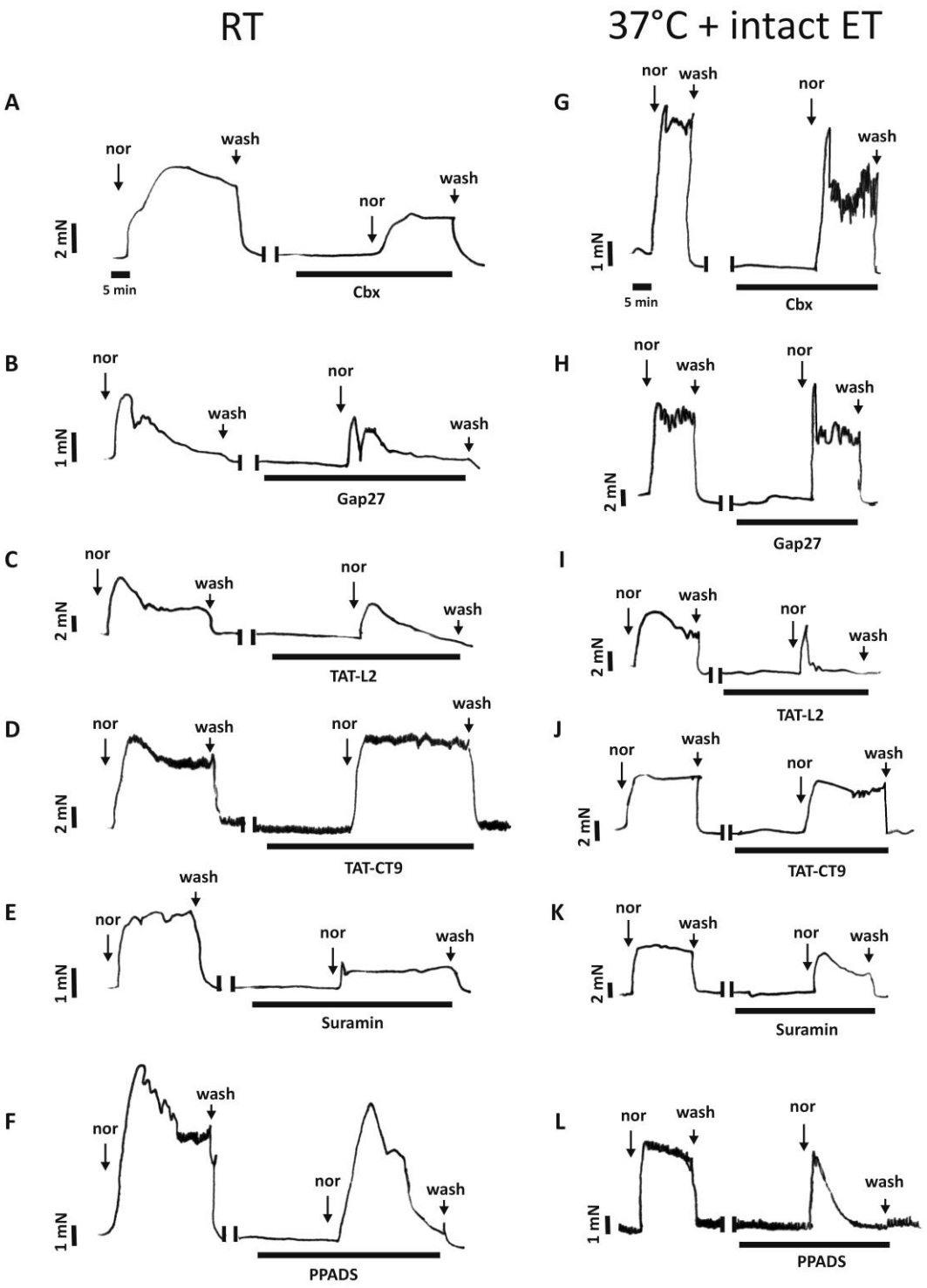


Figure 5. Representative mesenteric artery tension traces illustrating the influence of the various substances used on the contractile response induced by norepinephrine (nor) at room temperature (RT) without endothelium (A-F) and at 37° C with intact endothelium (ET) (G-L).

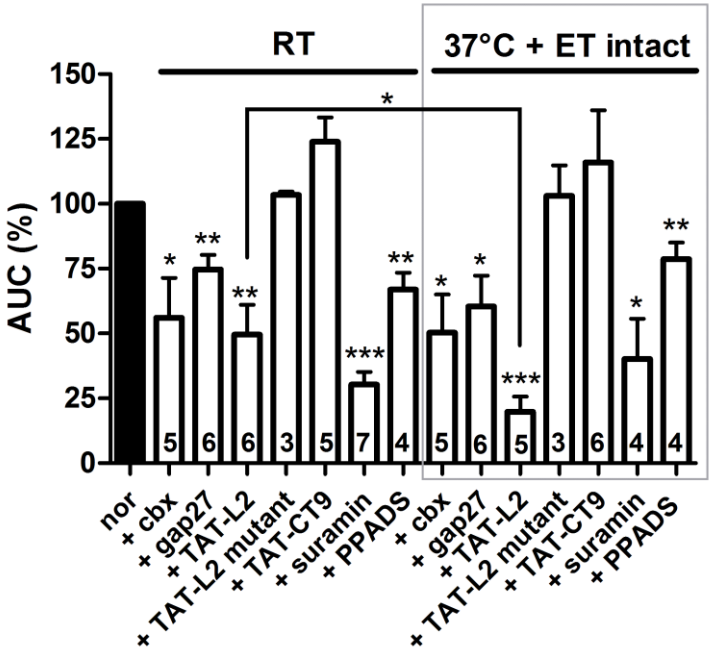


Figure 6. Summary data of the area under the curve (AUC) of the contraction curve for the various substances applied. The number of experiments is given in the bars. * p<0.05 **p<0.01 ***p<0.001.

DISCUSSION

The most salient result of the present work is the fact that TAT-L2, a peptide containing the L2 sequence of the cytoplasmic loop of Cx43, strongly reduces vasoconstriction of small mesenteric arteries triggered by norepinephrine. TAT-L2, as well as other connexin-targeting peptides, also inhibited norepinephrine-triggered Ca^{2+} oscillations in SMCs of isolated mesenteric arteries. Work in MDCK cells has suggested that Ca^{2+} entry *via* connexin hemichannels may contribute to agonist-induced Ca^{2+} oscillations (De Bock *et al.* 2012). Hemichannels are $[Ca^{2+}]_i$-controlled channels (de Vuyst *et al.* 2006, De Vuyst *et al.* 2009, Wang *et al.* 2012) that provide positive feedback to Ca^{2+} entry leading to Ca^{2+}-induced Ca^{2+}-entry. They also provide Ca^{2+}-induced ATP release (Braet *et al.* 2003, de Vuyst *et al.* 2006, De Vuyst *et al.* 2009) that will subsequently trigger P2X and P2Y receptor-mediated responses. Thus, inhibition of SMC Ca^{2+} oscillations by TAT-L2 is likely to be caused by blockage of

hemichannels, thereby disrupting Ca^{2+} entry and ATP release/purinergic signaling that may be crucial to support the Ca^{2+} oscillations (Figure 7) (Berridge 2007).

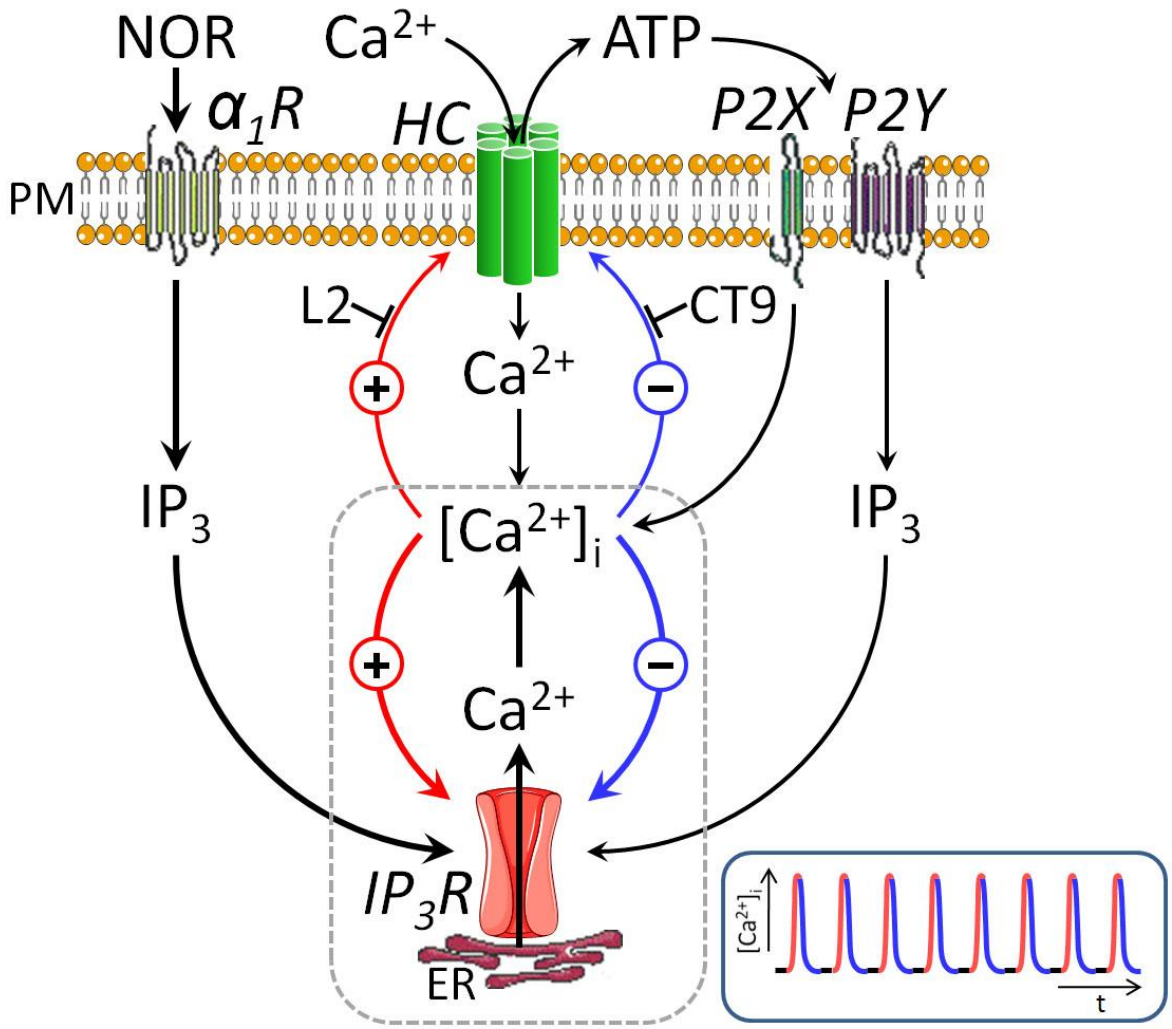


Figure 7. Schematic diagram illustrating the Ca^{2+} signaling aspects of this study. Norepinephrine (NOR) activates metabotropic α1-adrenergic receptors (α_1R) on the SMC plasma membrane (PM), resulting in IP_3 formation. IP_3 activates IP_3R opening with Ca^{2+} release from the endoplasmic reticulum (ER). Moderate $[Ca^{2+}]_i$ elevation promotes ER Ca^{2+} release (red arrow to IP_3R) while higher $[Ca^{2+}]_i$ levels inhibit Ca^{2+} release (blue arrow to IP_3R) and this is a basic mechanism leading to Ca^{2+} oscillations. The inset below illustrates the rising and falling phase of each Ca^{2+} spike, resulting from positive (red) and negative (blue) Ca^{2+} feed-back on IP_3Rs. Cx43 hemichannels (HC) are Ca^{2+}-permeable channels that are modulated by $[Ca^{2+}]_i$ in a similar manner as IP_3Rs, resulting in Ca^{2+}-induced Ca^{2+} entry at moderate $[Ca^{2+}]_i$ (red arrow to HC) and stop of Ca^{2+} entry at higher concentrations (blue arrow to HC). Hemichannel opening also triggers the release of ATP that activates P2X and P2Y receptors. SMC P2X1 receptors facilitate Ca^{2+} entry while P2Y receptors generate IP_3, and both sustain the central IP_3R-based oscillator (delineated by dotted grey line). Inhibition of positive and negative Ca^{2+} feed-back on Cx43 hemichannels with L2- and CT9-peptide respectively, inhibits the oscillations, suggesting a contribution of hemichannels in the oscillation mechanism; work with purinergic receptor antagonists and ATP-degrading enzymes supports the ATP signaling arm.

While moderate $[Ca^{2+}]_i$ elevation stimulates hemichannel opening, larger $[Ca^{2+}]_i$ increases result in hemichannel closure (de Vuyst *et al.* 2006, De Vuyst *et al.* 2009, Wang *et al.* 2012). This provides negative feedback that will stop Ca^{2+}-entry *via* hemichannels. CT9 peptide, identical to the last 9 amino acids of the C-terminal tail of Cx43, prevented high $[Ca^{2+}]_i$ closure of Cx43 hemichannels. CT9 peptide is identical to the αCT1 peptide (O'Quinn *et al.* 2011) that interferes with Cx43/ZO-1 interactions and thereby influences gap junction plaque size (Hunter *et al.* 2005). However, CT9 inhibition of high $[Ca^{2+}]_i$ hemichannel closure is independent of Cx43/ZO-1 interactions (De Bock *et al.* 2012). TAT-CT9 inhibited SMC Ca^{2+} oscillations, but by preventing high $[Ca^{2+}]_i$ –induced hemichannel closure, it also increased baseline $[Ca^{2+}]_i$. TAT-CT9 had no effect on norepinephrine-induced vasoconstriction, suggesting that the increased baseline Ca^{2+} signal might have compensated for the reduced spiking activity in driving SMC contraction.

The effects of carbenoxolone and Gap27 on Ca^{2+} spiking and SMC contraction were very comparable to TAT-L2 (at room temperature/without endothelium). Carbenoxolone rapidly inhibits both hemichannels and gap junction channels, while Gap27 first blocks hemichannels and with some delay (variable between cell types) also gap junctions (Braet *et al.* 2003, Decrock *et al.* 2009, De Bock *et al.* 2011, Wang *et al.* 2012). L2 peptide inhibits hemichannels while it slightly promotes junctional coupling by decreased dwelling of gap junction channels in the subconductance state (Seki *et al.* 2004). Recent work with a biologically active subdomain peptide of L2, gap19, gave similar results: no effect on gap junctions the first 30 min and slightly stimulating them after 24 h (Wang *et al.* 2013). Thus, carbenoxolone, Gap27 and TAT-L2 have in common that they inhibit hemichannels while having very divergent effects on gap junctions. Gap27 has previously been demonstrated to inhibit contractility of rat aorta (Tang and Vanhoutte 2008) and a combination of Gap27/gap26 suppressed vasomotion in rat mesenteric small arteries (Matchkov *et al.* 2006). In these cases, the effect was attributed to inhibition of gap junctions, either between SMCs, endothelial cells or as myoendothelial junctions. With the exposures time used here, Gap27 did not influence gap junctional coupling. The contraction experiments performed at room temperature/without endothelium were furthermore under conditions where endothelial and myoendothelial junctional coupling is grossly absent. Besides strongly different actions of L2 and Gap27 on gap junctions and hemichannels, another important

difference relates to their selectivity. Gap27 mimicks a sequence on the extracellular loop of the connexin protein that is well conserved between different connexins and therefore targets several connexins, including Cx37 and Cx43 present in mesenteric artery SMCs. By contrast, L2 peptide mimicks a sequence on the intracellular loop of Cx43 that is very different between different connexins. Its interaction partner is the C-terminal tail, that is also very different between different connexins. As a consequence, L2 peptide is expected to have better selectivity as compared to other connexin-mimetic peptides. Work with the L2-derived gap19 peptide has confirmed this notion and demonstrated no effect on hemichannels composed of Cx40 or Panx1 (Wang *et al.* 2013).

At 37 °C and intact endothelium, carbenoxolone and Gap27 had comparable effects on vessel contractility as recorded at room temperature/without endothelium. By contrast, TAT-L2 had a much stronger effect at 37 °C/intact endothelium. This may be related to a stronger cellular uptake of TAT-L2 at 37 °C or increased hemichannel activity with consequently larger effects upon inhibition. Alternatively, TAT-L2 promotion of gap junctional coupling may be stronger at 37 °C, thus favoring endothelial/myoendothelial Cx43-based junctional communication and promoting an endothelial vasodilatory influence.

Hemichannel opening is associated with ATP release (Kang *et al.* 2008) and interfering with purinergic signaling inhibited SMC Ca^{2+} oscillations and blood vessel contractility. An association between purinergic signaling and Ca^{2+} oscillations has been reported in other cell types (Geyti *et al.* 2008, De Bock *et al.* 2012), while a linkage with blood vessel contractility has been reported by Billaud et al. in thoracodorsal arteries (Billaud *et al.* 2011). Suramin and PPADs had comparable and profound inhibitory effects on Ca^{2+} spiking while suramin more strongly suppressed contractility. The spectrum of purinergic receptors targeted by both compounds is wide but discrete differences exist that may form the basis of this differential activity. For example, PPADS has most pronounced effects on metabotropic $P2Y_1$ and $P2Y_6$ while suramin does not influence $P2Y_4$ receptors (von Kugelgen 2006). Vascular smooth muscle cells express several P2Y receptors, including $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ that link to Ca^{2+} signalling (Govindan *et al.* 2010). However, SMCs also express P2X ionotropic receptors, more specifically $P2X_1$ receptors in SMCs of mesenteric blood vessels (Lamont *et al.* 2006), that facilitate Ca^{2+} entry upon activation (Lohman *et al.* 2012) and are inhibited by

suramin and PPADS (Lambrecht 2000). Thus, purinergic signaling may activate P2Y receptors leading to the generation of IP_3 while activation of $P2X_1$ receptors will facilitate Ca^{2+} entry. As a consequence, block of hemichannels with L2 peptide will reduce hemichannel Ca^{2+} entry and ATP release, turning down P2Y-associated IP_3 generation and shutting off $P2X_1$-related Ca^{2+} entry. Such targeting of Ca^{2+} signalling from two sides, Ca^{2+} entry and Ca^{2+} release, is likely to result in blockage of Ca^{2+} oscillations as observed here. ATP actions may be autocrine or paracrine, as for example in pancreatic β-cells where paracrine ATP signaling helps to coordinate Ca^{2+} oscillations in multiple cells (Hellman *et al.* 2004, Grapengiesser *et al.* 2005). Because we did not observe any synchronization of spiking events between cells, autocrine signaling is the more probable mode.

We did not find evidence for Panx1 in the presently used rat mesenteric artery SMCs, both at mRNA and protein level. By contrast, Billaud et al. (Billaud *et al.* 2011) have reported Panx1 expression in mouse thoracodorsal resistance artery SMCs and have furthermore demonstrated that Panx1 is expressed in SMCs of a variety of small diameter resistance vessels (Lohman *et al.* 2012). Our results in rat mesenteric arteries differ from these observations, in terms of expression as well as function, as ^{10}panx1 peptide did not influence the Ca^{2+} oscillations. Possibly, this is related to differences in vascular beds (mesenteric versus thoracodorsal) and species (rat versus mouse). Notwithstanding these differences, the results obtained here may well apply to the case of Panx1 channels present in other vascular beds.

In conclusion, our results show that TAT-L2 peptide strongly inhibits norepinephrine-induced contraction of mesenteric arteries, indicating a role of Cx43 hemichannels and purinergic signaling in the control of vascular tone. Cx43 hemichannels thus come up as a novel target to interfere with vascular function, and TAT-L2 peptide adds as a new tool to modulate blood vessel function in the context of vascular disease.

Acknowledgements

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Chapter VII: Inhibiting connexin channels protects against cryopreservation-induced cell death in human blood vessels

Inhibiting connexin channels protects against cryopreservation-induced cell death in human blood vessels

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Abstract

Objectives: Cryopreserved blood vessels are being increasingly employed in vascular reconstruction procedures but freezing/thawing is associated with significant cell death that may lead to graft failure. Vascular cells express connexin proteins that form gap junction channels and hemichannels. Gap junction channels directly connect the cytoplasm of adjacent cells and may facilitate the passage of cell death messengers leading to bystander cell death. Two hemichannels form a gap junction channel but these channels are also present as free non-connected hemichannels. Hemichannels are normally closed but may open under stressful conditions and thereby promote cell death. We here investigated whether blocking gap junctions and hemichannels could prevent cell death after cryopreservation.

Materials and Methods: Inclusion of Gap27, a connexin channel inhibitory peptide, during cryopreservation and thawing of human saphenous veins and femoral arteries was evaluated by TUNEL assays and histological examination.

Results: We report that Gap27 significantly reduces cell death in human femoral arteries and saphenous veins when present during cryopreservation/thawing. In particular, smooth muscle cell death was reduced by 73% in arteries and 71% in veins, while endothelial cell death was reduced by 32% in arteries and 51% in veins.

Conclusions: We conclude that inhibiting connexin channels during cryopreservation strongly promotes vascular cell viability.

Key words: connexin, cryopreservation, cell death, blood vessel grafting

INTRODUCTION

Vascular grafts have wide surgical applications but the preservation of those grafts has faced several problems. Grafts are preserved in cold storage at 4 °C as well as cryopreserved, but the procedure is still unsatisfactory and, after grafting, complications such as thrombosis and vasospasms occur which could lead to late graft failure (Callow 1996). The risk for thrombosis is enhanced by rejection, leading to loss of endothelial lining and function, and in a later phase, by immune related intimal hyperplasia and fibrosis (Vermassen *et al.* 1994). Even with the introduction of immunosuppressive treatment, the improvement of harvesting techniques and preservation fluids, and the use of anticoagulation therapy the patency of these allografts was not improved in most studies (Randon *et al.* 2010).

The performance of cryopreserved vascular grafts is suboptimal, mainly because of spontaneous vessel wall fractures (Bujan *et al.* 2000) appearing at the time of thawing or grafting. Another important issue is that the current methods of vascular cryopreservation used in most vascular banks lead to a certain degree of loss of the intimal endothelial layer (Pascual *et al.* 2004). Before arterial or venous grafts are implanted to bypass occluded coronary arteries they undergo extensive apoptotic and necrotic cell death in the intimal and medial layers (Kouzi-Koliakos *et al.* 2007). Cell death plays an important role in vascular graft failure, and important for the present study, cell-cell communication may act to expand the cell death process to neighboring healthy cells, leading to bystander cell death during cryopreservation and thawing (Decrock *et al.* 2009).

The most direct form of cell–cell communication is provided by gap junctions that consist of intercellular channels composed of connexin proteins, named according to their predicted molecular weight (Saez *et al.* 2003). Vascular cells abundantly express connexins, with Cx37, Cx40 and Cx43 as the most representative isoforms (Haefliger *et al.* 2004). Gap junction channels are formed by the interaction of two hemichannels, belonging to the membranes of adjacent cells and directly connect the cytoplasm of neighboring cells (Figure. 1). Gap junctions are able to pass cell death messengers leading to bystander cell death (Decrock *et al.* 2009). Recent work from our group has demonstrated that the physiological messenger IP_3, which can pass through gap junction channels, becomes a crucial cell death-communicating messenger under pro-apoptotic conditions (Decrock *et al.* 2012). Additionally, hemichannels, which are half gap junction channels, may by themselves

promote cell death. Unapposed hemichannels in the plasma membrane are typically closed and only open when they become incorporated into a gap junction. However, unapposed hemichannels not assembled into gap junctions may open under certain conditions like cell depolarization, decreased extracellular calcium, changes in intracellular calcium concentration, alterations in phosphorylation or redox status, mechanical strain, and ischemic and inflammatory conditions (Wang *et al.* 2012). Hemichannels are non-selective channels that allow, like gap junctions, the passage of up to 1.5 kDa molecules; as a consequence, uncontrolled hemichannel opening may lead to cell death, caused by excessive entry of sodium and calcium ion and loss of cellular ATP or other crucial metabolic molecules (Retamal *et al.* 2007, Decrock *et al.* 2009).

Gap junction channels and hemichannels can be inhibited by connexin mimetic peptides such as Gap26 and Gap27 (de Wit and Griffith 2010, De Bock *et al.* 2011, Wang *et al.* 2012). These peptides mimic a sequence on the extracellular loops of the connexin protein and first inhibit unapposed hemichannels, that have their extracellular loops freely available for peptide interactions, followed by a somewhat delayed inhibition of gap junctions (Decrock *et al.* 2009, Evans *et al.* 2012, Wang *et al.* 2012). Previous work has demonstrated that connexin mimetic peptides prevent the propagation of cell death by inhibiting both gap junctions and (unapposed) hemichannels (Decrock *et al.* 2009). Recent work from others has furthermore shown that these peptides can significantly improve the outcome after experimental ischemia applied to heart or brain (Hawat *et al.* 2010, Davidson *et al.* 2012).

Here, we used Gap27, which targets the two vascular connexins Cx37 and Cx43 (Haefliger *et al.* 2004), to investigate the hypothesis that the combined inhibition of gap junctions and hemichannels exerts a cell protective effect during the exposure of donor blood vessels to the stressful events of cryopreservation and thawing (Figure 1). Our results show that Gap27 significantly reduces cell death in endothelial cells (ECs) and smooth muscle cells (SMCs) of cryopreserved human blood vessels. The strongest effects of Gap27 were found in SMCs of both vessel types and in the ECs in the veins. We further found that veins have a better conserved intimal layer compared to arteries and have less cell death in the ECs.

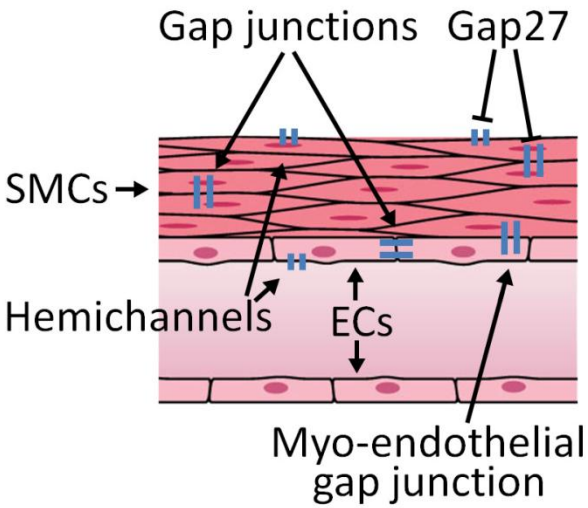


Figure 1. Diagram of a bloodvessel illustrating the hypothesis investigated in this paper. Vascular cells express connexins that form gap junctions and hemichannels. Gap junctions connect the cytoplasm of smooth muscle cells (SMCs) and of endothelial cells (ECs); they also connect ECs with SMCs (Myo-endothelial gap junctions). Gap junctions can communicate cell death from dying to healthy neigboring cells, resulting in bystander cell death. Hemichannels are normally closed but may open in response to mechanical strain, ischemia, inflammation, low extracellular calcium, depolarization and altered phosphorylation or redox status. This can lead to the loss of essential metabolites and ionic shifts over the plasma membrane, thereby promoting cell death. Gap27 inhibits hemichannels within minutes and gap junctions with some delay; when present for several hours, both gap junctions and hemichannels will be inhibited. We investigated whether inclusion of Gap27 in the media used for cryopreservation and thawing could protect against vascular cell death.

MATERIALS AND METHODS

Tissue preparation

Tissue samples of human femoral artery and saphenous vein were obtained from deceased donors (age: 45 $\pm$ 1.7 years, n=13) according to a protocol that was approved by the ethical committee of the University Hospital Ghent. Blood vessels were selected and procured according to the Belgian law and guidelines of the Superior Health Council Belgium. Exclusion criteria were: presence of ulcers, calcific atheromas, rupture of intima or media and traction lesions due to prelevation in arteries; signs of phlebitis, atheromatosis, parietal abnormalities and lesions of intima in veins. Serologic testing for human immunodeficiency virus, hepatitis B and C and syphilis was also done and vessels were excluded in case of positivity. One centimeter long vessel samples were placed in HBSS-HEPES and prior to cryopreservation, DMSO was added to a final concentration of 15 %. DMSO is the most commonly used cell membrane-permeating freeze point lowering agent for cryopreservation

of blood vessels (Muller-Schweinitzer 2009) and concentrations up to 15% were shown to be safe for that purpose (Song *et al.* 1995). In Gap27 treated vessels, the peptide was added to the HBSS-HEPES-DMSO solution at a final concentration of 200 µM. Grafts were cryopreserved at a rate of 1 °C/min in a -80 °C freezer and after two weeks stored for at least another two weeks in a liquid nitrogen container (-196 °C).

For vessel analysis and cell death studies, tissues were thawed in a 37 °C water bath and kept on ice before the next steps. DMSO was then progressively washed out by a stepwise reduction of the DMSO concentration in the HBSS-HEPES solution. To that purpose, the blood vessels remained for 4 min in HBSS-HEPES solution with respectively 10 %, 5 %, 2.5 % and 0 % DMSO. In Gap27 treated vessels, the peptide (200 µM) was present during DMSO washout.

As shown in Figure 5A, we also performed experiments to verify the effect of Gap27 when included during cryopreservation/thawing only or during DMSO washout only.

After DMSO washout, blood vessels were transferred to a neutral buffered 4 % formalin solution for fixation during 24 h and were then embedded in paraffin. Paraffin-embedded vessel specimens were cut into 5 µm serial sections along a transverse plane and were then stained with haematoxylin and eosin for microscopic analysis. Images were taken on a Nikon TE300 inverted microscope using a x10 or x20 objective and equipped with a Nikon DS-Ri1 cooled color CCD camera (Nikon Belux, Brussel, Belgium).

Immunostaining

Paraffin slices were deparaffinized and subsequently incubated with an antigen retrieval solution (Vector, Lab Consult, Brussels, Belgium). Preincubation with a blocking buffer (Bovine serum albumin, 5 mg/ml; bovine skin gelatin, 0.1 %; Triton X-100, 0.25 % in phosphate-buffered saline (PBS)) was done for 30 min at room temperature. Sections were incubated overnight at 4 °C with primary antibody (anti-Cx43 polyclonal AB (1/250, Sigma, Belgium), anti-Cx37 polyclonal (1/100, Zymed, Life Techologies) or anti-Cx40 polyclonal (1/10, Thermo Fisher, Belgium) diluted in blocking solution. After washing, a secondary antibody conjugated to Alexa-594 (1/500, Molecular Probes, Life Technologies) was added. The sections were mounted with an antifade solution containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Lab Consult) for nuclear staining. Images were acquired on

a Nikon TE300 inverted epifluorescence microscope with a x20 objective and camera as referred to before.

Apoptosis assay

Embedded artery or vein sections were deparaffinized, rehydrated through graded alcohol, and permeabilized with 0.1 % Triton X-100 at room temperature (8 min incubation). Slides were rinsed twice in a phosphate-buffered saline (PBS). Apoptosis was detected through in situ terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL), using the In Situ Cell Death Detection Kit (Roche, Vilvoorde, Belgium). Slides were incubated with the TUNEL reaction mixture containing TdT and fluorescein-dUTP for 1 hr at 37 °C; thereafter, they were rinsed three times in PBS and mounted as described for immunostaining. Control experiments performed on C6 glioma cells stably transfected with Cx43 exposed to staurosporine (2 µM, 6 hours) induced massive TUNEL positivity while exposure to the DMSO vehicle had no effect. TUNEL-stained blood vessel tissue sections were examined by epifluorescence microscopy as described for immunostaining. Four areas from each slide were examined and 5 slides per blood vessel were analyzed. The number of TUNEL-positive cells is given as a percentage of the total number of cells quantified from DAPI positive counts.

Pharmacological agents

Gap27 (SRPTEKTIFII) and scrambled Gap27 (TFEPIRISITK) were synthesized by Thermo Fisher Scientific (Ulm, Germany) at >80% purity. HBSS-HEPES (Gibco, Merelbeke, Belgium) contained (in mM) $CaCl_2$, 1.26; $MgCl_2$-$6H_2O$, 0.493; $MgSO_4$-$7H_2O$, 0.407; KCl, 5.33; KH_2PO_4, 0.441; $NaHCO_3$, 4.17; NaCl, 137.93; Na_2HPO_4, 0.338; D-Glucose (Dextrose), 5.56; Phenol Red, 0.0266. PBS contained (in mM) NaCl, 137; KCl, 2.68; KH_2PO_4, 1.47; $Na_2HPO_4.2H_2O$, 6.46; pH 7.4. Staurosporine was obtained from Sigma (Bornem, Belgium).

Statistical Analysis

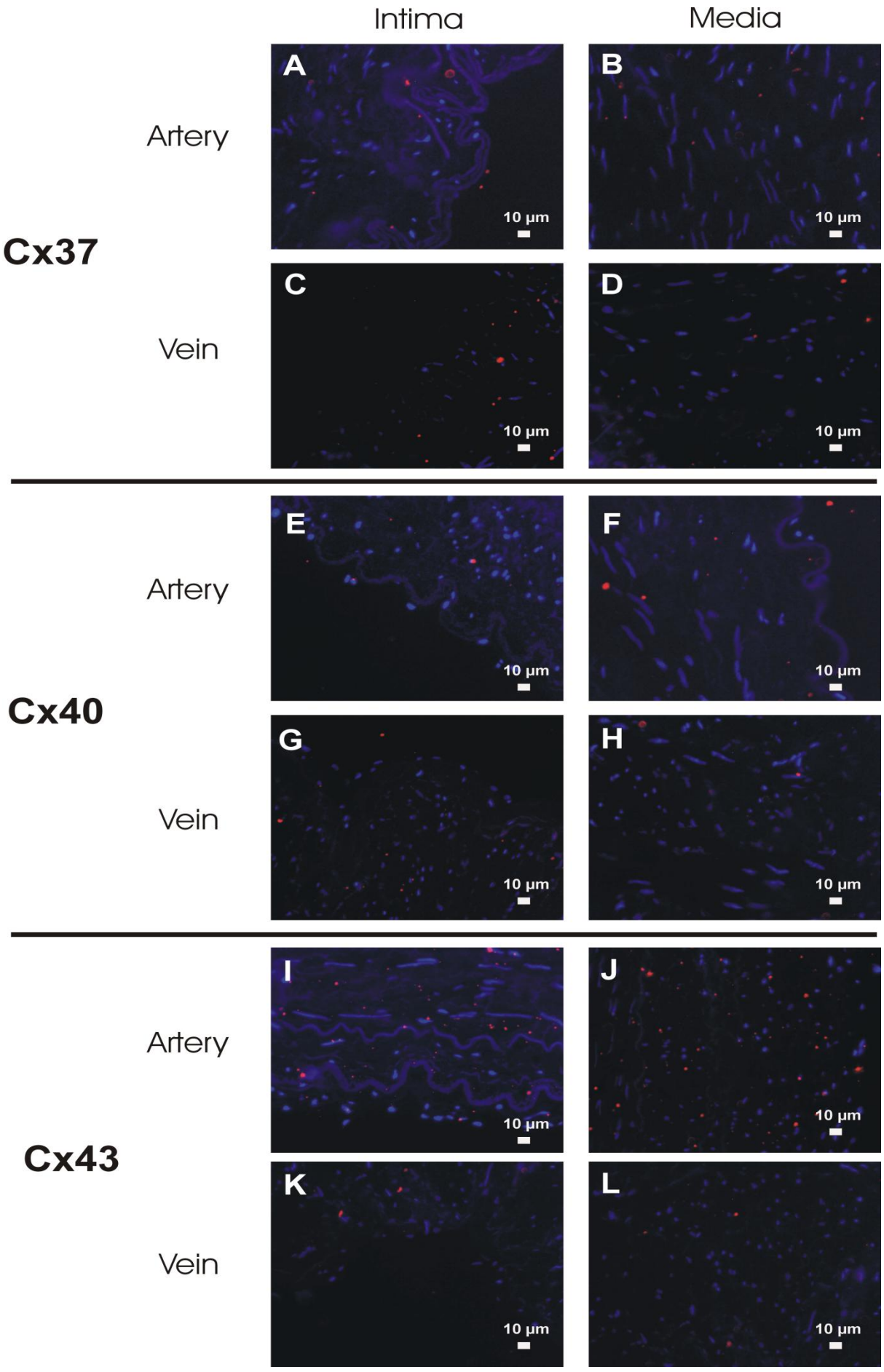
All data are expressed as mean ± SEM. Statistical analysis between groups was performed using Friedman Test with Dunn's post-t-test. Proportions were compared using Pearson's Chi-square test. Differences in cell death between control conditions of both blood vessels are analyzed by unpaired Student's t-test. Correlations were assessed by Pearson's Correlation tests. A value of $P < 0.05$ was considered as statistically significant. The symbol *n* represents the number of blood vessels.

RESULTS

Connexin expression in human femoral arteries and saphenous veins

Immunostainings demonstrated the presence of the major vascular connexins, Cx37, Cx40 and Cx43 in the intimal and medial layers of human cryopreserved blood vessels. Cx37 was mainly found in the intimal EC layer of saphenous veins, while it was present in intimal EC and medial SMC layers in femoral arteries (Figure 2A-D). Cx40 was found in both arteries and veins and was present in the intimal and medial layers (Figure 2E-H). Cx43 was most abundantly present and was mainly located in the medial layer of femoral arteries and in intimal and medial layers of saphenous veins (Figure 2I-L).

Figure 2. Connexin expression in human saphenous veins and femoral arteries. A-D. Representative example images illustrating Cx37 immunostainings (red) overlayed on nuclear DAPI stainings (blue) in endothelial cells in the intimal and smooth muscle cells in the medial layer of femoral arteries (Artery) and saphenous veins (Vein). E-H. Example images illustrating Cx40 expression. I-L. Example images illustrating Cx43 expression.


Intima
Media
Artery
Vein
Cx37
Cx40
Cx43
A
B
C
D
E
F
G
H
I
J
K
L
10 µm

Intimal layer detachment after cryopreservation is more important in femoral arteries than in saphenous veins

Morphological analysis of haematoxylin-eosin stained blood vessel sections showed that femoral arteries had a pronounced detachment of the intimal lining compared to saphenous veins (Figure 3A-D). Quantification of the number of blood vessels with an intact intimal layer, less than 50 % intimal loss and more than 50 % loss, allowed us to construct a graph depicting the distribution pattern of loss of intimal integrity (Figure 3C-D). These data showed a significantly different distribution pattern between femoral arteries and saphenous veins, with a clearly better conserved intimal lining in the latter. When Gap27 (200 µM) was included during cryopreservation, thawing and DMSO washout, the number of vessels with more than 50 % intimal loss was decreased but this visible difference did not attain statistical significance (Figure 3C-D). We verified whether intimal loss was correlated with the time between vessel procurement from the donor and the start of cryopreservation but found no significant correlation.

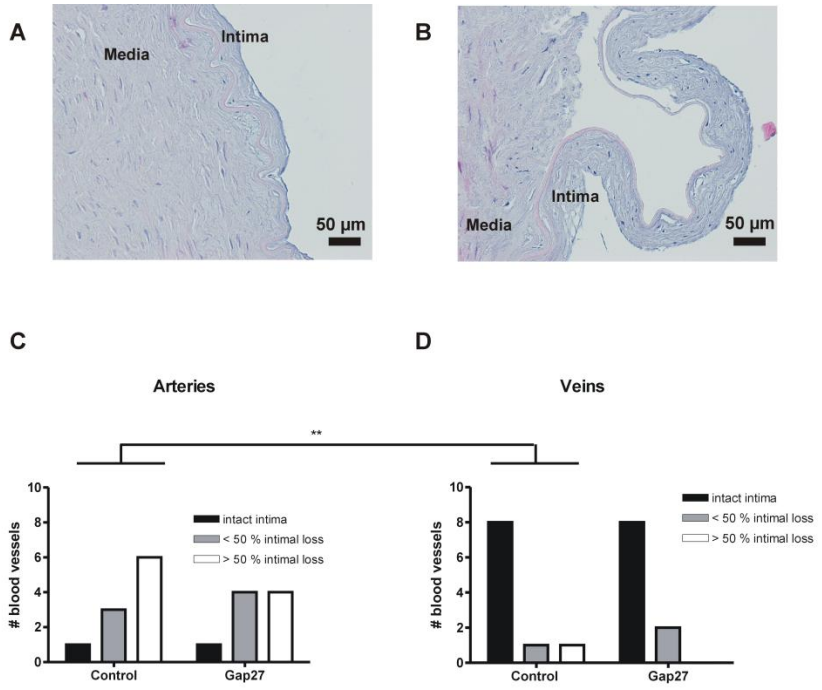


Figure 3. Intimal layer detachment in femoral arteries and saphenous veins. A-B. Example images illustrating an intact (A) and detached intimal layer (B) in two different femoral arteries. C-D. Summary data illustrating intimal loss distribution patterns of femoral arteries (C) and saphenous veins (D). The intimal layer was significantly better conserved in saphenous veins than in femoral arteries (** P <0.01).

Gap27 reduces apoptotic cell death in cryopreserved human blood vessels

In order to quantify cell death in cryopreserved/thawed blood vessels, we performed TUNEL stainings to detect apoptotic cell death. As can be appreciated from Figure 4A&C, non-treated blood vessels displayed clearly discernable TUNEL positivity in the intimal and medial layers. We verified whether the degree of TUNEL positivity varied with the duration of the time interval between vessel procurement and start of cryopreservation but found no significant correlation. Interestingly, we found that Gap27 (200 µM) visibly reduced TUNEL positivity counts when it was present during cryopreservation, thawing and DMSO washout. Average data of several such experiments are given in Figure 5. In femoral arteries, ~27 % of the ECs and ~5 % of the SMCs were TUNEL positive under control conditions. Gap27 significantly reduced these cell death counts by more than 1/3 in the intimal layer and by more than 2/3 in the medial layer (Figure 5A&C). In saphenous veins, TUNEL positivity was ~14 % in ECs and ~6 % in SMCs. Gap27 significantly reduced these counts by almost 1/2 in ECs and by more than 1/2 in SMCs (Figure 5B&D). Gap27 with a scrambled peptide sequence had no significant effects on cell death counts (Figure 5). Thus, Gap27 more than halved cell death in SMCs while cell death reduction was less than half in ECs.

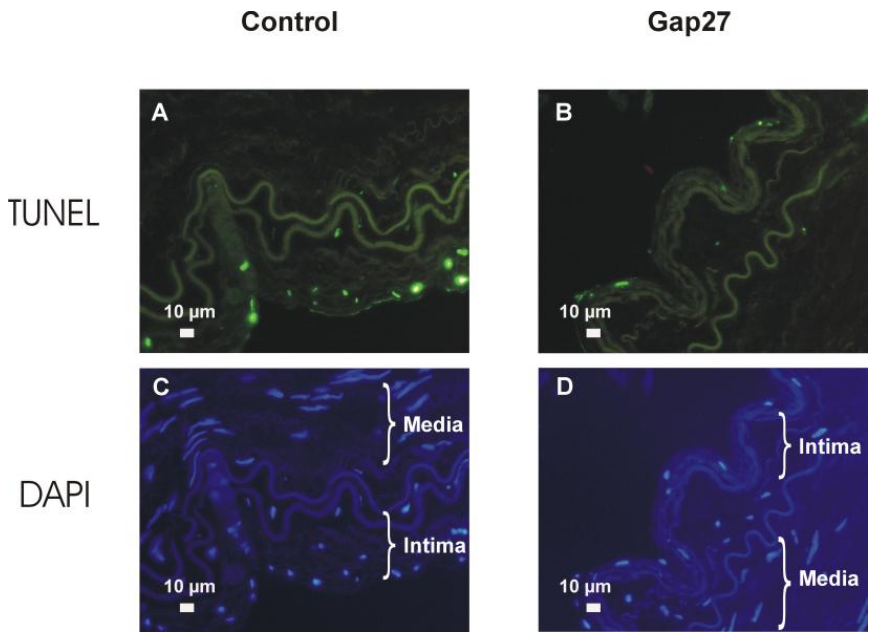


Figure 4. Gap27 reduces apoptotic cell death in EC and SMC layers in cryopreserved human blood vessels. A-D. Example images of TUNEL (A-B, green) and DAPI (C-D, blue) stained sections of femoral arteries under control and after Gap27 treatment. The number of TUNEL positive cells was visibly reduced by Gap27 in ECs and SMCs from intimal and medial layers respectively.

Cell death in control (without Gap27) was much more prominent in ECs than in SMCs (ECs: 27 and 14 % in arteries and veins respectively vs. SMCs: 5 and 6 %) and, as a consequence, the fraction of cells rescued by Gap27 may be more important in ECs as compared to SMCs. We therefore calculated the protective effect of Gap27 relative to the total number of cells (EC or SMC) as an alternative to calculations relative to the number of dead cells. Table 1 summarizes these two measures of Gap27 protection and demonstrates that the cell mass rescued by Gap27 was larger in ECs than in SMCs. Note that the numbers given in Table 1 were calculated from the combined data of figure 5 and figure 6 and therefore slightly differ from the relative effect size described above in relation to figure 5.

Comparison of the control bars in panels A and B of figure 5 suggests that EC death is more prominent in arteries than in veins; panels H and I of figure 6 (presented below) indicate a similar difference. Table 2 summarizes average results in control conditions from the combined dataset of figure 5 and 6 with extra data from additional experiments, and shows that EC death was significantly lower in veins as compared to arteries.

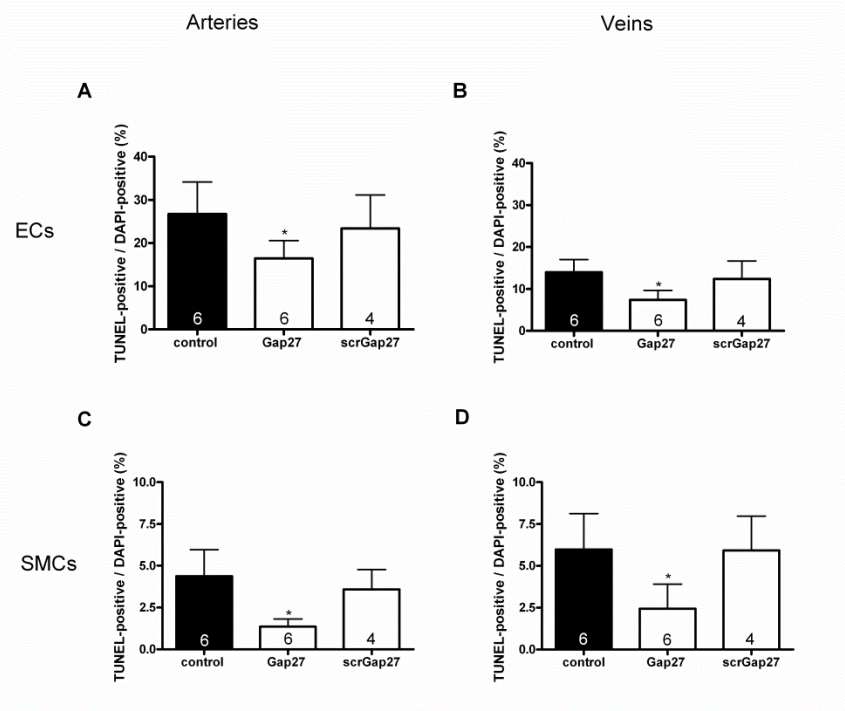


Figure 5. Gap27 reduces apoptotic cell death in cryopreserved human blood vessels – average data. Apoptotic cell death in ECs (A, B) and SMCs (C, D) of femoral arteries (A, C) and saphenous veins (B, D). The number of TUNEL positive cells was significantly reduced by Gap27 in ECs and SMCs. Gap27 with a scrambled peptide sequence had no significant effects on cell death counts. The numbers of vessels are indicated in the bars. * $P < 0.05$

In a next step, we determined in which phase Gap27 exerted most of its protective effects: during cryopreservation/thawing or during DMSO washout. Thus, we investigated cell death with Gap27 being included either during cryopreservation/thawing or during DMSO washout (Figure 6A). We found that inclusion of Gap27 during DMSO washout-only, slightly (but non-significantly) reduced cell death in ECs and SMCs while inclusion during cryopreservation/thawing-only always gave larger and statistically significant protective effects in both femoral artery and saphenous vein. Figure 6B-G illustrates example images of TUNEL and DAPI stainings while average data are given in figure 6H-K.

Table 1. Protective effect of Gap27 on cell death in ECs and SMCs.

	Protection relative to total number of dead cells (%)		Protection relative to total number of cells (%)	
	Arteries	Veins	Arteries	Veins
EC	32 ± 8 (n=11)	51 ± 8 (n=13)	11 ± 4 (n=11)	12 ± 4 (n=13)
SMC	73 ± 7 (n=11)	71 ± 6 (n=13)	6 ± 3 (n=11)	6 ± 2 (n=13)

Average values express inhibitory effect based on Gap27 treatment data of figure 5 and 6 (Figure 6 condition with Gap27 present during cryopreservation/thawing).

Table 2. Vascular cell death in femoral arteries and saphenous veins.

	Arteries	Veins
EC	30 ± 5 % (n=15)	17 ±3 % * (n=17)
SMC	7 ± 3 % (n=15)	7 ± 2 % (n=17)

Average values based on control condition data of figure 5, 6 and additional experiments.
* significantly below value for arteries with P <0.05.

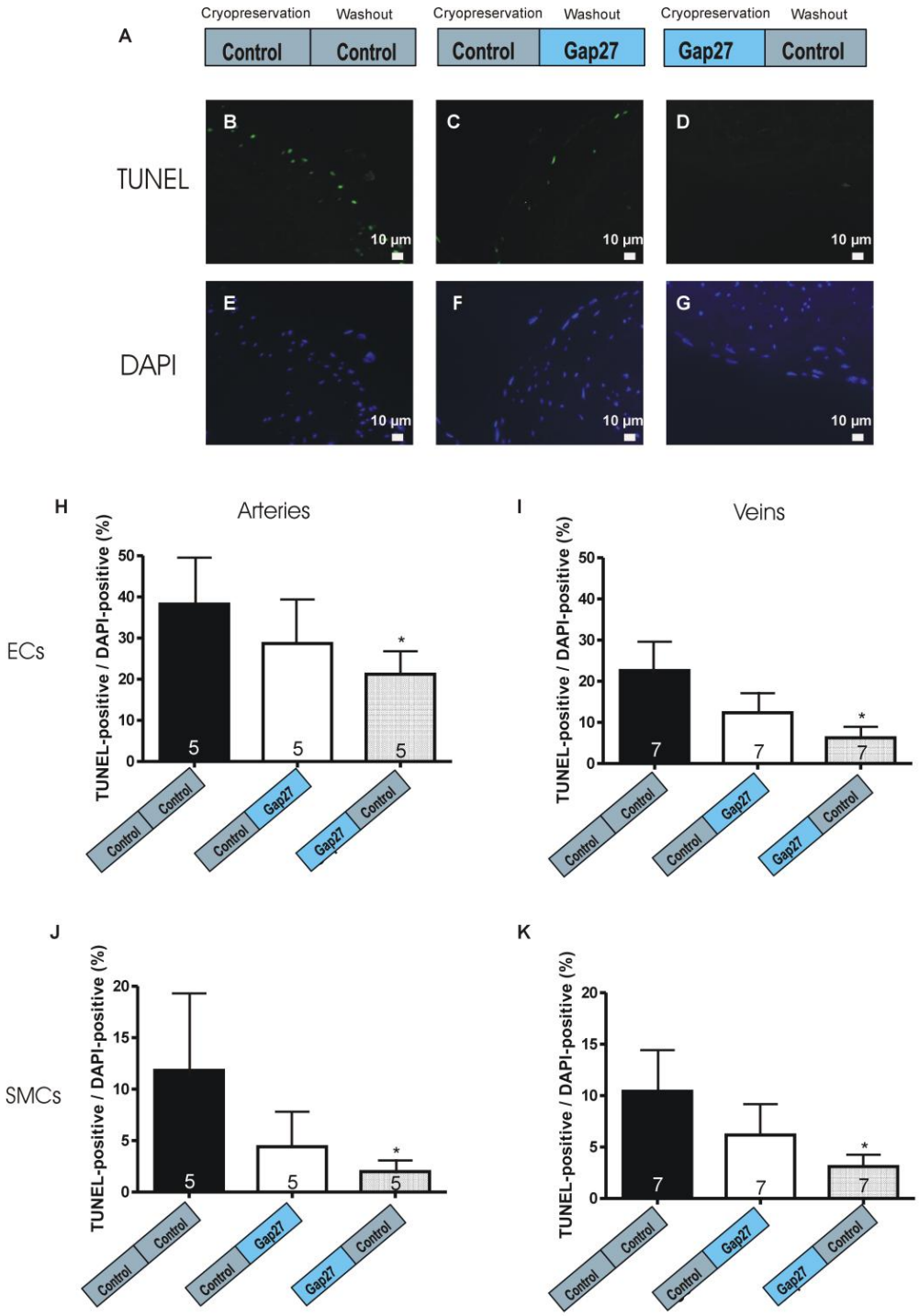


Figure 6. Gap27 exerts most of its protective effect when added during cryopreservation/thawing. A. Overview of the different treatment protocols used. 'Cryopreservation' refers to Cryopreservation/thawing and 'washout' to DMSO washout. B-D. Example images of TUNEL stainings (green) in a saphenous vein. E-G. Corresponding example images of nuclear DAPI staining (blue) in the same preparation. Treatment was as indicated in the horizontal bars above each image (A). H-K. Average data of experiments as illustrated in A-G. Inclusion of Gap27 during DMSO washout-only, slightly (but non-significantly) reduced cell death in ECs and SMCs while inclusion during cryopreservation/thawing-only always gave larger and statistically significant protective effects in both femoral artery and saphenous vein preparations. Numbers of vessels are indicated in the bars. * $P < 0.05$

DISCUSSION

This study demonstrates that inhibition of connexin channels (gap junctions and hemichannels) with the connexin mimetic peptide Gap27 that targets Cx37 and Cx43, significantly reduces cell death of SMCs and ECs in cryopreserved and subsequently thawed human femoral arteries and saphenous veins. Gap27 had slight but non-significant effects when it was present during DMSO washout only and most of its protective effect appeared to be related to its presence during cryopreservation/thawing.

Cryopreserved vessels are being increasingly employed in vascular reconstruction procedures (Pascual *et al.* 2004). The considerable cell death provoked by cryopreservation is a major factor leading to the post-transplantation failure of vascular allografts (Pascual *et al.* 2004). In the present study, apoptotic cell death was more pronounced in the intimal layer than in the medial layer. Apoptosis in the ECs of vascular grafts has been extensively addressed in previous reports (Holzknecht *et al.* 2002, Pascual *et al.* 2004, Kanellaki-Kyparissi *et al.* 2005) and the influence of alternative procedures for prelevation, cryopreservation and thawing have been scrutinized (Bujan *et al.* 2000, Pascual *et al.* 2004). When compared to the application of a more gradual, automated thawing process yielding a 20 % reduction in EC death (Pascual *et al.* 2004), protection by Gap27 appears to be stronger. It remains to be determined whether the combination of these treatments, i.e. automated slow thawing plus inclusion of Gap27, yields an additive effect. Automated thawing is, however, not available as a standard procedure in all hospitals. Compromised endothelial preservation can lead to vasospasms, occlusive intimal hyperplasia and accelerated atherosclerosis (Steen 2001). Other reports have indicated the occurrence of apoptosis and SMC loss in the tunica media (Hirsch *et al.* 1998, Kanellaki-Kyparissi *et al.* 2005) which, together with intimal proliferative lesions can lead to chronic rejection of the arterial allograft (Hirsch *et al.* 1998).

Gap junctions form the most direct cell-cell communication pathway, connecting the cytoplasm of adjacent cells. They have a central role in vascular physiology, helping in coordinating the contraction of SMCs (Figueroa and Duling 2009), communicating electrical and Ca^{2+} signals in upstream direction *via* the endothelium (Dora 2010) and contributing to the fine tuning of EC-SMC communication *via* myo-endothelial gap junctions. Additionally,

gap junctions allow the passage of cell death messenger signals, thereby potentially contributing to the propagation of cell death known as bystander cell death (Decrock *et al.* 2009). Work of our lab has recently demonstrated that the physiological messenger IP_3 can turn into a crucial cell death messenger under pro-apoptotic conditions (Decrock *et al.* 2012). While gap junctions may cause propagated bystander cell death, unapposed hemichannels may cause cell death by uncontrolled opening thereby contributing to the entry and escape from the cell of ions and small molecules below ~1.5 kDa (Decrock *et al.* 2009). Work from our lab has demonstrated that this effect may additionally contribute to the propagation of cell death (Decrock *et al.* 2009).

We here demonstrate the presence of vascular connexins Cx37, Cx40 and Cx43 in human femoral arteries and saphenous veins. Previous studies have reported that connexin expression varies with vessel type, species and even vessel diameter (Haefliger *et al.* 2004). These studies indicated Cx37 expression primarily in the intima, Cx40 in both intima and media and a prominent expression of Cx43 typically in the medial SMC layer. Our results grossly support this pattern with the exception that Cx37 was also found (although not prominently) in the media of human arteries and Cx43 in the intima of human veins (again much less prominent than in the media). We used Gap27 that mimics the second extracellular loop of both Cx37 and Cx43, to inhibit gap junctions and hemichannels (Decrock *et al.* 2009, de Wit and Griffith 2010, De Bock *et al.* 2011).

Our results show a clear decrease of cell death in the intimal and medial layers of arteries and veins treated with Gap27. The smaller effect of Gap27 on EC death of arteries (Figure. 5A and Table 1) may be related to a lower Cx43 expression in this layer. Interestingly, the gap junction and hemichannel inhibitors carbenoxolone and Gap26 were previously reported to reduce neointimal formation and this led to a reduced SMC deterioration and decreased risk for atherosclerosis and other complications (Song *et al.* 2009). In addition to this, it is important to realize that Gap27-reduced SMC death may be beneficial for ECs because the two cells are coupled *via* myo-endothelial gap junctions (Figure 1) that may pass cell death messengers from SMCs (the largest vascular cell population) to ECs. Conversely, a reduction by Gap27 of EC death may be beneficial at the functional level after transplantation given the important role of these junctions in endothelial control over SMC

function (Dora 2010). Previous work has demonstrated that cryopreservation of arterial specimens in low calcium solutions is associated with (unexpected) increased cell death (Bujan *et al.* 2000). Low extracellular calcium conditions are known to trigger hemichannel opening (Thimm *et al.* 2005) and it is tempting to speculate that this is one of the causes leading to increased vascular cell death. Thus, combining low extracellular calcium and Gap27 would be an interesting option to be explored in follow-up work.

Gap27 exerted most of its protective effects during cryopreservation/thawing, while it was far less effective when only present during DMSO washout after thawing. Earlier reports have indicated that rapid thawing increases the fragility of the arterial vessel wall and leads to spontaneous macro- and microfractures in the vessel wall due to thermal stress created by temporal and spatial temperature gradients (Pegg *et al.* 1997, Bujan *et al.* 2000). It was earlier reported that the procedure of vascular cryopreservation used at most vascular banks provoke a certain loss of the endothelium, which is one of the main factors leading to graft failure (Pascual *et al.* 2004). Our work demonstrates that detachment of the endothelial layer was more often observed in arteries than in veins; also EC death was more pronounced in arteries than in veins. Previous reports have demonstrated that slow thawing is better suited for arterial grafts (Ruddle *et al.* 2000); thus, the prominent detachment of endothelial layer in arteries may be related to the less optimal thawing procedure used in our experiments. However, since slow thawing is not generally available in most hospitals, venous grafts, with a better preserved endothelium and therefore better antithrombotic potential, should be recommended for patients with severe vascular disease and with a higher risk for thrombosis.

In conclusion, inhibition of the vascular connexins Cx37 and Cx43 with Gap27 peptide significantly protects human blood vessels against cell death that occurs in relation to the cryopreservation/thawing-procedure. Gap27 contains a sequence identical to an extracellular facing domain of Cx37 and Cx43 and will thus not act immunogenically when some residual peptide would be left over after thorough washing. Further studies including clinical application of the peptides will be needed to determine whether the protective potential of this treatment translates into a clinically better outcome after graft vessel transplantation in patients.

Acknowledgments

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Chapter VIII: General discussion and perspectives

In this work we explored the modulation of blood vessel function by methanandamide, CGRP and CxHCs in small mesenteric rat arteries (Figure 1). Connexin channels (GJs and HCs) are important for the physiology of blood vessel function but may also contribute to cell death under pathological conditions. A specific form of pathological condition is the cell stress associated with cryopreservation and thawing. Thus in a next move, we investigated whether inhibiting GJs and HCs could improve the viability of cryopreserved human blood vessels (Figure 1).

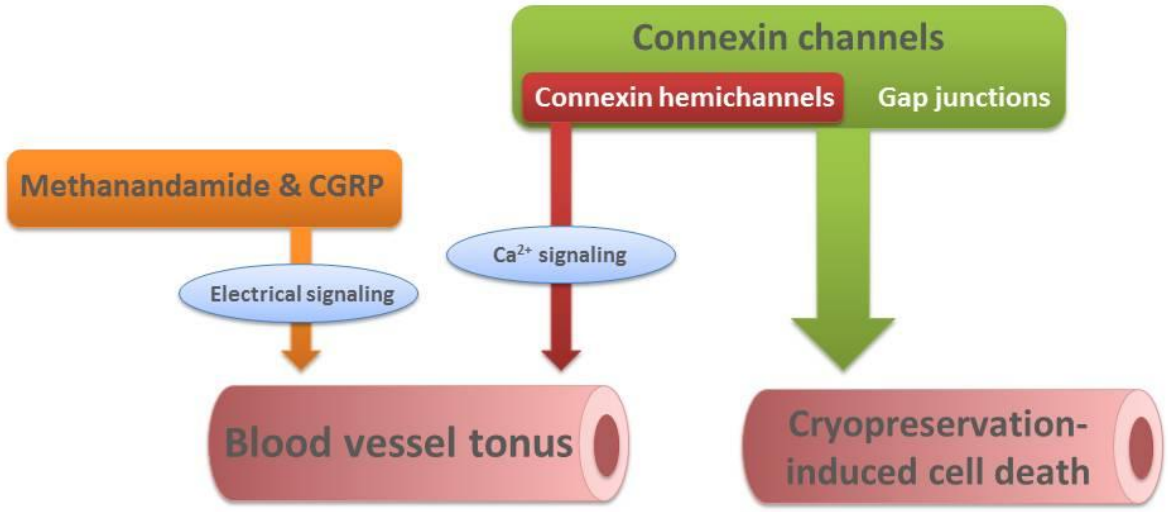

Figure 1. Overview of the topics we addressed in this doctoral thesis. We investigated the modulation of the blood vessel function by methanandamide and the perivascular nerves-releasing factor CGRP and CxHCs in small mesenteric rat arteries. Connexin channels may also contribute to cell death under pathological conditions such as cell stress associated with cryopreservation and thawing of human blood vessels. We investigated whether inhibiting GJs and HCs could improve the viability of these cryopreserved vascular grafts.

Endocannabinoids, such as anandamide, have potent vasodilatory actions in a variety of vascular preparations. The mechanism of action of these endocannabinoids is complex and vary with species, vessel type, and even vessel size (Vanheel and Van de Voorde 2000, O'Sullivan *et al.* 2004). Several studies have found that anandamide or methanandamide stimulate TRPV1 (instead of the classical CB1 receptors) thereby releasing CGRP (Ralevic *et al.* 2000, Ahluwalia *et al.* 2003). The mechanism involved in vasorelaxation to CGRP seems to vary among species and vessel types. In some vessels, CGRP acts *via* an endothelium dependent mechanism, whereby CGRP interacts with endothelial receptors stimulating the release of endothelium derived relaxing factors (Gray and Marshall 1992, Wisskirchen *et al.*

1998, Boussery *et al.* 2005). By contrast in other vessel types, such as small mesenteric rat arteries, CGRP acts directly on the smooth muscle cells by increasing the cAMP content (Ishikawa *et al.* 1993, Yoshimoto *et al.* 1998). The resultant activation of PKA has been shown to open K^+ channels. K_{ATP} channels as well as BK_{Ca} channels were shown to be involved in this vasorelaxing effect of CGRP dependent on the vessel type and contractile condition.

In **chapter V** we specifically addressed the question whether CGRP might activate BK_{Ca} currents in small mesenteric rat artery SMCs since previous studies in our lab showed that vasorelaxation to CGRP was partly sensitive to low concentration of TEA (Breyne *et al.* 2006). In contrast to previous studies, we used acutely isolated myocytes to investigate the direct effects of methanandamide and CGRP on these cells. Our findings showed that methanandamide failed to increase membrane K^+ currents and failed to hyperpolarize the membrane potential of SMCs of small mesenteric arteries. Moreover, we found that CGRP is acting specifically and directly on these myocytes by increasing the BK_{Ca} channel activity *via* activation of the CGRP receptor and *via* cAMP- and PKA-dependent pathways. This suggests that methanandamide relaxes and hyperpolarizes intact rat mesenteric arteries by releasing CGRP from perivascular nerves. Methanandamide decreased the K^+ currents and this was inhibited by the K_V channel blocker 4-AP. This was in accordance with a previous study, showing a similar inhibition of K^+ current in aortic SMCs (Van den Bossche and Vanheel 2000) and strongly argues against a direct hyperpolarizing influence of methanandamide on SMCs from small mesenteric arteries. Application of exogenous CGRP substantially increased mesenteric SMC K^+ currents and hyperpolarized the membrane potential. The controversy in literature concerning the type of K^+ channel activated by CGRP could be explained by the contractile tone of the artery. The activation of K^+ channels by CGRP can be dependent on the choice of initial experimental conditions under which the vessel is placed. In precontracted rat coronary arteries, BK_{Ca} channel blockers attenuated the CGRP-induced reduction in both tension and $[Ca^{2+}]_i$. K_{ATP} channel blockers did not affect the CGRP-induced responses in these precontracted vessels. In resting arteries, only pretreatment with the combination of BK_{Ca} and K_{ATP} channel blockers attenuated the CGRP-induced decrease in $[Ca^{2+}]_i$ and tension (Sheykhzade and Nyborg 2001).

In conclusion, blood vessel tonus can be modulated by methanandamide *via* the release of CGRP from perivascular nerves leading to a hyperpolarization and vasorelaxation.

Next to electrical changes, vessel diameter and blood flow are also controlled by Ca^{2+} signals. Intracellular Ca^{2+} mediates a variety of vascular endothelial and smooth muscle cell functions. Smooth muscle cells respond to biological activators with oscillatory and propagating rises in $[Ca^{2+}]_i$ that are highly organized in both time and space. GJs play a crucial role in the communication between vascular cells and in the synchronization of Ca^{2+} signals thereby tightly controlling the level of vasoconstriction. Before being incorporated into GJs, CxHCs reside in the plasma membrane in a closed state. Such unapposed non-junctional HCs can be opened by various messengers and conditions, thereby forming a pore that allows the passage of ATP and ions. CxHC opening is regulated by $[Ca^{2+}]_i$ and previously our lab showed that the opening of CxHCs crucially contributes to Ca^{2+} oscillations by providing Ca^{2+} entry that is necessary to sustain oscillatory activities (De Bock *et al.* 2011).

In **chapter VI** we investigated the role of CxHCs on SMC $[Ca^{2+}]_i$ responses and contractility of small mesenteric arteries. We suggest that HCs and ATP signaling are involved in controlling SMC contraction and blood vessel diameter by influencing the Ca^{2+} oscillations. The HC contribution appears to be essential as inhibition with connexin mimetic peptides stops the oscillations and inhibits SMC contraction.

Ca^{2+} oscillations are influenced by HC opening and closure with increases in the $[Ca^{2+}]_i$, in a similar way as the dependency of IP_3-receptor channel opening probability on $[Ca^{2+}]_i$. CxHCs are activated by $[Ca^{2+}]_i$ changes (de Vuyst *et al.* 2006, Ponsaerts *et al.* 2010) and are thus expected to open with each SMC Ca^{2+} spike and to release ATP in the extracellular space. ATP can bind to autocrine purinergic metabotropic receptors and the subsequent release of IP_3 can contribute to the oscillations. Additionally, CxHC opening may favor Ca^{2+} entry (Saez *et al.* 2010), which is necessary for sustained Ca^{2+} oscillations (Alonso *et al.* 1999). Thus, block of hemichannels with connexin mimetic peptides will reduce ATP-induced IP_3 generation and Ca^{2+} entry, thereby stopping the Ca^{2+} oscillations. In addition to autocrine actions of ATP, these actions may as well be paracrine. However, because no synchronization of oscillations was observed, paracrine signaling must be absent. In the tension measurements we saw in intact arteries sometimes vasomotion appear. In this circumstances, paracrine signaling of ATP could contribute to vasomotion. Furthermore, in those tension measurements, the effect of the connexin mimetic peptide Gap27 and the TAT-L2 peptide on the contractility was as inhibitory as in vessels without vasomotion, pointing to a role for CxHCs in vasomotion as well. This would mean that besides the very

well described role of GJs in vasomotion, CxHCs should be considered as additive players in this mechanism. Future studies should investigate thoroughly the role of CxHCs in vasomotion. Certainly since upregulation of vasomotion is considered to be protective in pathological conditions such as hypertension and ischemia.

Gap27 and the TAT-L2 peptide inhibit the opening of CxHCs and this leads to a reduction of the norepinephrine-induced Ca^{2+} oscillations and contractility. The TAT-CT9 peptide inhibits the closure of CxHCs with high micromolar $[Ca^{2+}]_i$ and the effect of this peptide is a persistent opening of the CxHC, and in this way the negative feedback loop is inactive and Ca^{2+} oscillations are inhibited. An additional effect of this prolonged opening of the HCs is the entrance of Ca^{2+} into the cytosol through these channels and this explains why the baseline Fluo-3 fluorescence increased in this condition and why the contractility in our blood vessels did not attenuate with the TAT-CT9 peptide.

Changes in $[Ca^{2+}]_i$ modulate vascular SMC tension (Ruehlmann *et al.* 2000). There is increasing evidence that pharmacological and/or pathophysiological factors modulate SMC responsiveness *via* alterations in the Ca^{2+} oscillations. NO, NO donors, β-adrenoceptor agonists, intravenous anesthetics, or benzodiazepines reduce the frequency of Ca^{2+} oscillations along with inducing relaxation (Savineau and Marthan 2000). Gap27 and the TAT-L2 peptide attenuated the norepinephrine-induced contractions in our isometric tension measurements. Cx mimetic peptides have been extensively used to investigate the role of GJs in vascular physiology (Dhein 2002) but our results support that the effect of these peptides on connexin channels should be reconsidered in the sense that these peptides also, and more rapidly, inhibit unapposed CxHCs. In addition to the well documented functions of GJs in controlling vascular contraction, our data bring up a possible contribution of CxHCs. These novel targets offer interesting opportunities for the development of new therapeutic approaches to vascular disease. Future experiments on hypertensive animal models are necessary to determine whether CxHCs are also involved in this particular disease state. It would be interesting to test the effects of the L2-peptide and Gap27 in spontaneous hypertensive rats (SHR rats) or in hypertensive animal models induced by angiotensin II or the nitric oxide synthase inhibitor L-NAME. *In vivo* studies of blood pressure in those animals treated with the connexin mimetic peptides would further elucidate the therapeutic potential of these substances. These peptides could be used as another target in the prevention of hypertension.

GJs and CxHCs are important for vascular function, but connexin channels have also been described as contributors to the cell death process. GJs allow the passage of cell death messenger signals, thereby potentially contributing to the propagation of cell death known as bystander cell death or 'kiss of death' (Frank *et al.* 2005, Vinken *et al.* 2006, Decrock *et al.* 2009, Feine *et al.* 2012). A positive correlation between GJIC and apoptosis was suggested since carbenoxolone and other GJ blockers prevented apoptosis (Frank *et al.* 2005, Peixoto *et al.* 2009). While GJs may cause propagated bystander cell death, unapposed HCs may cause cell death by uncontrolled opening thereby contributing to the entry and escape from the cell of ions and small molecules below ~1.5 kDa (Decrock *et al.* 2009). This may additionally contribute to the propagation of cell death (Decrock *et al.* 2009).

Cryopreserved vessels, employed in vascular reconstruction procedures (Pascual *et al.* 2004), show massive cell death provoked by the methods of vascular cryopreservation. This is one of the main factors leading to the failure of grafting procedures performed using cryopreserved vascular allografts (Pascual *et al.* 2004).

In **Chapter VII** we explored the possibilities of blocking connexin channels by Gap27 in the prevention of cryopreserved-induced cell death in vascular grafts. We showed a clear decrease of cell death in the intimal and medial layers of arteries and veins treated with Gap27. Song et al. stated earlier that carbenoxolone and Gap26 reduced neointimal formation and SMC deterioration and decreased risk for atherosclerosis and other complications (Song *et al.* 2009). Since SMCs are coupled with ECs *via* myo-endothelial GJs, cell death messengers from SMCs may transfer to ECs. In this way, Gap27-reduced SMC death may be beneficial for ECs. On the other hand, a reduction by Gap27 of EC death may be beneficial as well, since these myo-endothelial junctions play an important role in endothelial control over SMC function (Figueroa and Duling 2009, Dora 2010). Preservation of viable endothelial cells has the advantage of also preserving the antithrombogenic properties of the endothelium, which are important in the avoidance of early graft thrombosis, especially in low-flow situations (Randon *et al.* 2010). Partial absence of the endothelium means that the extracellular matrix makes contact with the circulating blood, which generally leads to thrombosis and/or restenosis (Pascual *et al.* 2004). On the other hand, preservation of viable endothelium has the disadvantage that the immunogenicity of the allografts is also preserved because vascular endothelial cells express ABO and major histocompatibility complex class I and II antigens. An immune response by activated T-

lymphocytes after implantation of an allograft due to ABO incompatibility was a significant risk factor for patency (Carpenter and Tomaszewski 1998). This immune reaction can be counteracted by matching the ABO and by administering a low dose of immunosuppressants.

We used Gap27 that mimics the second extracellular loop of both Cx37 and Cx43, to inhibit GJs and HCs (Evans and Leybaert 2007, Decrock *et al.* 2009, de Wit and Griffith 2010, De Bock *et al.* 2011, Wang *et al.* 2012). In order to determine whether HCs or GJs are the main contributors to cell death in cryopreserved vascular grafts; application of Gap19, which has specificity for Cx43 and only inhibits HCs, could elucidate this question. Although the effects of the Gap19 peptide might be reduced due to blocking of Cx43 HCs, and not Cx37 HCs.

In order to improve the preservation of vascular grafts, several cryopreservation solutions were tested over the years. Some authors tried cryopreservation of arterial specimens in low Ca^{2+} solutions but this was associated with increased cell death (Bujan *et al.* 2000). Low $[Ca^{2+}]_e$ conditions are known to trigger HC opening (Quist *et al.* 2000, Thimm *et al.* 2005) and it is tempting to speculate that this is one of the causes leading to increased vascular cell death. The protective effects of Gap27 were more convincing during cryopreservation/thawing, while it was far less effective when only present during DMSO washout after thawing. Previous studies have shown that rapid thawing increases the fragility of the arterial vessel wall and macro- and microfractures in the vessel wall occur due to thermal stress (Pegg *et al.* 1997, Bujan *et al.* 2000). In this study, apoptotic cell death was more pronounced in the intimal layer than in the medial layer. Bad endothelial preservation can lead to vasospasm, occlusive intimal hyperplasia and accelerated atherosclerosis (Steen 2001). Applying a more gradual, automated thawing process yields a 20 % reduction in EC death (Pascual *et al.* 2004) and a combination of gradually automated thawing (1 °C/min) and connexin mimetic peptides could be even more beneficial to reduce cell death in the endothelial layer. However, in most hospitals (in Belgium) automated thawing is not a standard procedure.

In this study we demonstrated that detachment of the endothelial layer was more often observed in arteries than in veins; also EC death was more pronounced in arteries than in veins. Thus, veins have a better preserved endothelium after cryopreservation as compared to arteries and this property should be taken into account as one of the criteria in the decision which vascular graft to use for a particular patient. A possible explanation for the

worse preservation of the endothelium of arteries could be that both blood vessels are flushed prior to cryopreservation and since arteries have a more 'robust' structure compared to veins, flushing might be less carefully done (with higher pressure). Veins are more fragile and flushing happens here with more care to not damage the vein. Therefore a consideration of the flushing technique in arteries might improve endothelial cell survival.

In this study, we focused on cell death studies and histological examination of cryopreserved vascular grafts after application with the connexin mimetic peptide. Future experiments are necessary to assess the more functional properties of the blood vessels *via* isometric tension measurements for example. The contractility induced by norepinephrine could be tested and the endothelium-dependent vasodilation could be investigated by acetylcholine. An evaluation by this method was already done by Rigol *et al.* in cryopreserved porcine femoral arteries, showing a 50 % reduction in norepinephrine-induced contractions and an equal dilation compared to control non-cryopreserved blood vessels (Rigol *et al.* 2000). It would be interesting to test our blood vessels cryopreserved with Gap27 and evaluate if the vascular functions were improved compared to standard cryopreserved grafts.

Further studies including clinical application of the peptides will be needed to determine whether the protective potential of this treatment translates into a better clinical outcome after vessel transplantation. A first follow up study could be done by transplanting two blood vessels, one treated with Gap27 and one control, into pigs and procure the vessels after several weeks. A histological examination of the blood vessels would already indicate if certain adverse effects are developing. Major effects such as thrombosis, rejection, etc, are mostly seen after 6 months to 1 year of transplantation. Follow-up studies on patients would be a second step and here we could investigate the long-term effects of implanting cryopreserved Gap27-incubated grafts and see if adverse effects such as intimal hyperplasia, rejection of the graft or infection is reduced in this population.

This newly proposed procedure, cryopreservation combined with application of connexin mimetic peptides, could be expanded to other tissues as well and for tissues that were vitrified (e.g. oocytes) The effects we found in blood vessels could be extrapolated to cryopreserved heart valves, bone tissues, keratinocytes, etcetera. Therefore additional studies testing the impact of blocking connexin or pannexin channels during cryopreservation/thawing of those tissues could improve the outcome after transplantation. Addition of connexin mimetic peptides could also be beneficial in preservation solutions for

non-cryopreserved tissues such as cornea, heart, liver,…. The cornea for example, is stored mostly at 2-8 °C in North America or in organ culture at 28-37 °C in Europe, leading to a prolonged preservation time. The successful outcome of the majority of corneal transplants depends on the presence of a viable corneal endothelium (Armitage 2011). Corneas express several connexin isoforms such as Cx43 (in endothelium and epithelium)(Williams and Watsky 2002) and Cx50 (in epithelium) (Gatzioufas *et al.* 2008), and blocking those connexins could reduce cell death and improve viability of the grafts. Bone fragments express Cx43 abundantly and blocking this connexin by gap peptides could be important for preserving tissues such as ear bones (hammer or anvil) (Batra *et al.* 2012). Application of mimetic peptides could improve the preservation of organs and tissues and thereby contributing to a better outcome after transplantation.

To conclude, in this doctoral thesis we elucidated the modulation of methanandamide, CGRP and CxHCs on the blood vessel function and we investigated the beneficial effect of connexin channel blockers on cryopreservation-induced cell death in vascular grafts (Figure 1).

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Curriculum Vitae

Personalia

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Cohabiting, 2 children

Education

Specific teacher training (2004-2006, Ghent University)

Master of Science in Biomedical and Clinical Engineering – option Artificial Organs (2002-2004, Ghent University)

Master of Science in Revalidation Therapy and Physiotherapy (1998-2002, Ghent University)

Latin-Mathematics (1992-1998, Sint-Jan Berchmanscollege, Avelgem)

Publications

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Bol M, Vanheel B. Influence of methanandamide and CGRP on whole cell K^+ currents in acutely isolated mesenteric artery smooth muscle cells. Belgian Society of Fundamental and Clinical Physiology and Pharmacology; Liège, Belgium; November 3, 2008. Poster presentation

Bol M, De Bock M, De Vuyst E, Wang N, Decrock E, Van Moorhem M, Vanheel B, Leybaert L. Hemichannels contribute to Ca^{2+} dynamics in smooth muscle cells in acutely isolated small mesenteric arteries. Belgian Society of Fundamental and Clinical Physiology and Pharmacology; Brussels, Belgium; October 24, 2009. Poster presentation

Bol M, Vanheel B, Leybaert L. Modulation of vascular function by cannabinoids, CGRP and connexin hemichannels. PhD Day: Basic Medical Sciences, Ghent, Belgium; May 25, 2009. Oral presentation

Bol M, De Bock M, De Vuyst E, Wang N, Decrock E, Monsalvo J, Decaluwé K, Vanheel B, Van de Voorde J, Leybaert L. Hemichannel involvement in Ca^{2+} dynamics and contractility of smooth muscle cells in acutely isolated small mesenteric arteries. Belgian Society of Fundamental and Clinical Physiology and Pharmacology; Namur, Belgium; March 27, 2010. Poster presentation

Bol M, De Bock M, De Vuyst E, Wang N, Decrock E, Decaluwé K, Vanheel B, Van de Voorde J, Leybaert L. Hemichannels contribute to Ca^{2+} dynamics and contractility of smooth muscle cells in acutely isolated small mesenteric arteries. International Gap Junction Conference 2011; Ghent, Belgium; August 6-11, 2011. Poster presentation

Bol M, De Bock M, De Vuyst E, Wang N, Decrock E, Decaluwé K, Vanheel B, Van de Voorde J, Leybaert L. Hemichannels contribute to Ca^{2+} dynamics and contractility of smooth muscle cells in acutely isolated small mesenteric arteries. Mini-symposium: connexin-hemichannels from structure-function to therapeutic applications; Leuven, Belgium; August 22, 2011. Oral presentation

Bol M, De Bock M, De Vuyst E, Wang N, Decrock E, Decaluwé K, Vanheel B, Van de Voorde J, Leybaert L. Connexin hemichannels contribute to Ca^{2+} dynamics and contractility of smooth muscle cells in acutely isolated small mesenteric rat arteries. Interuniversitary Attraction Poles (IUAP); Ghent, Belgium; December 16, 2011. Poster presentation

Bol M, Breyne J, Leybaert L, Vanheel B. Influence of CGRP and methanandamide on membrane potential, K^+ currents and calcium oscillations in mesenteric artery smooth muscle. EDHF 2012; Vaux-de-Cernay, France; June 27-30, 2012. Poster presentation

Bol M, De Bock M, De Vuyst E, Wang N, Decrock E, Decaluwé K, Vanheel B, Van de Voorde J, Leybaert L. Connexin hemichannels are involved in Ca^{2+} dynamics and contractility of SMC in small mesenteric rat arteries. 12th Symposium of the European Calcium Society on Calcium-binding proteins in normal and transformed cells; Toulouse, France; September 8-12, 2012. Poster presentation

Training

Doctoral Training Programme Health Sciences and Medicine:

Specialist courses:

Basic Course in Laboratory Animal Science Partim I: General Topics (2007)
Basic Course in Laboratory Animal Science Partim II: General Topics (2007)
Biosafety seminar (2008)
Clinical studies: study design, implementation and reporting (2012)
Biosafety seminar (2012)

Seminars in transferable skills:

Cluster career management:
- *'Het feedbackgesprek' (2009)*
- *Basisassistententraining' (2009)*
- *From PhD to Job Market (2nd doctoral conference) 2010*
- *Knowledge for growth 2011*

Cluster communication skills: - *Effective scientific communication (2010)*

Cluster leadership and personal efficiency: - *Leading, Following and Collaborating (2011)*

Educational support

Practical courses:

Spirometry (2nd Bach Pharmaceutical Sciences; 2nd Bach Dentistry; 2nd Bach Biomedical Sciences; 1st Bach Logopedics and Audiology)

Blood pressure (2nd Bach Pharmaceutical Sciences; 1st Bach Logopedics and audiology, 2nd Bach Biomedical Sciences)

Muscle-nerve apparatus (2nd Bach Dentistry)

Reflexes (1st Bach Biomedical Sciences; 2nd Bach Medicine)

Oxygen consumption (1st Bach Logopedics and Audiology, 2nd Bach Dentistry)

Eye (2nd Bach Medicine)

Smooth muscle (1st Bach Biomedical Sciences)

PC-electrophysiology (1st Bach Medicine and Dentistry)

Internships Neurophysiology (1st Mas Biomedical Sciences)

Neurovascular coupling

Cytoplasmic calcium and vascular function

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